Approach to a Synthetic Esterase

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Contents

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Finally I wish to pay a special thanks to my family and Helene for their support and encouragement throughout.

Declaration

I hereby declare that this thesis is based solely on my own work unless otherwise stated. In the case of collaboration, the nature of it is indicated in the main body of the thesis and the researchers involved are stated.

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Abstract

The work described in this thesis covers two projects:

a) The development of a supported sixteen residue peptidyl model incorporating the 'reactive triad' serine, histidine and aspartic acid, found within serine protease enzymes. The methodology was designed to allow rapid screening of a combinatorial library of simple peptides containing the reactive triad, some of which were anticipated to show synthetic esterase activity. It is envisaged that this could be undertaken by observing selective cleavage of a highly coloured red azo dye tethered through the ester under investigation to the peptide *via* a glycol linker. One peptidyl system has been synthesised and investigated to allow the development of the methodology and it was found that intramolecular hydrolysis could be observed when glycol linkers of optimal length were utilised under favourable conditions.

b) Vancomycin was modified with extended glycol chains containing a terminal double bond to allow covalent dimerisation *via* cross coupling metathesis. Binding of the vancomycin monomers and dimers to an *N*-acetyl-L-Lys-D-Ala-D-Ser peptide model has been demonstrated *via* electrospray ionisation mass spectrometry by Professor Albert Heck at Utrecht, Holland and *in-vivo* studies are ongoing to elucidate if enhanced vancomycin activity towards vancomycin resistant bacteria can be observed.

Abbreviations

Ar	aromatic
Boc	tertiary-butoxycarbonyl
BOP	benzotriazole-1yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate
Bu	butyl
brs	broad singlet
CCM	cross coupling metathesis
CI	chemical ionisation
COSY	correlated spectroscopy
d	doublet
Da	dalton
DAP	diaminopimelic acid
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DCC	dicyclohexylcarbodiimide
dd	doublet doublet
DEAD	diethyl azodicarboxylate
DIPEA	diisopropylethylamine
DMAP	4-(dimethylamino) pyridine
DNA	deoxyribonucleic acid
E	enzyme
EI	electron impact
eq.	equivalent

ESI-MS	electrospray ionisation mass spectroscopy
FAB	fast atom bombardment
Fmoc	9-fluorenylmethoxycarbonyl
GISA	glycopeptide-intermediate *S. aureus*
HBTU	2-(N-benzotriazole-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate
HMPA	hexamethylphosphotriamide
HMQC	heteronuclear multiple quantum coherence
HOBt	N-hydroxybenzotriazole
HPLC	high performance liquid chromatography
Hz	hertz
IR	infrared
J	coupling constant
LC	liquid chromatography
LDA	lithium diisopropylamide
m	multiplet
M	molar solution
(M)	parent ion
Me	methyl
MIC	minimum inhibitory concentration
mmol	milli molar
mp	melting point
Ms	mesyl group
MS	mass spectrometry
NMO	4-methylmorpholine N-oxide

NMR	nuclear magnetic resonance
p	para
PBP	penicillin-binding protein
PDC	pyridinium dichromate
PEG	polyethylene glycol
Ph	phenyl
PPh_3	triphenylphosphine
ppm	parts per million
PyBOP	Benzotriazole-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate
PyBROP	bromo-tris-pyrrolidino-phosphonium hexafluorophosphate
q	quartet
s	singlet
S	substrate
SPPS	solid phase peptide synthesis
t	triplet
TBAF	tetrabutylammonium fluoride
TBDPS	tertiary-butyldiphenylsilyl
TBS	*t*-butyldimethylsilyl
TBSCl	tertiary-butyldimethyl chloride
TBTU	2-(N-benzotriazole-1-yl)-1,1,3,3,-tetramethyluronium tetrafluoroborate
tert	tertiary
TFA	trifluoroacetic acid
TFMSA	trifluoromethanesulfonic acid

THF	tetrahydrofuran
TIPS	triisopropylsilyl
TIS	triisopropylsilane
TLC	thin layer chromatography
TMS	tetramethylsilane
TNBS	2,4,6-trinitrobenzenesulfonic acid
TPAP	tetra-n-propylammonium perruthenate
Ts	tosyl group
UV	ultra violet
μmol	micro molar

1 Introduction

1.1 Enzymes

1.1.1 History

For many thousands of years since the time of ancient Egypt, mankind has been using biological enzymes in the fermentation process. An example of this was brewing beer.[1] Ever since, our ancestors have tried to rationalise the 'invisible or vital force' responsible for converting a sweet and sticky mash into a palatable liquid bearing the gift of euphoria. An excellent historical account of the rational and heated debates that lead to the elucidation of the 'vital force' being attributed to enzymes, has been provided by Dressler and Potter.[1]

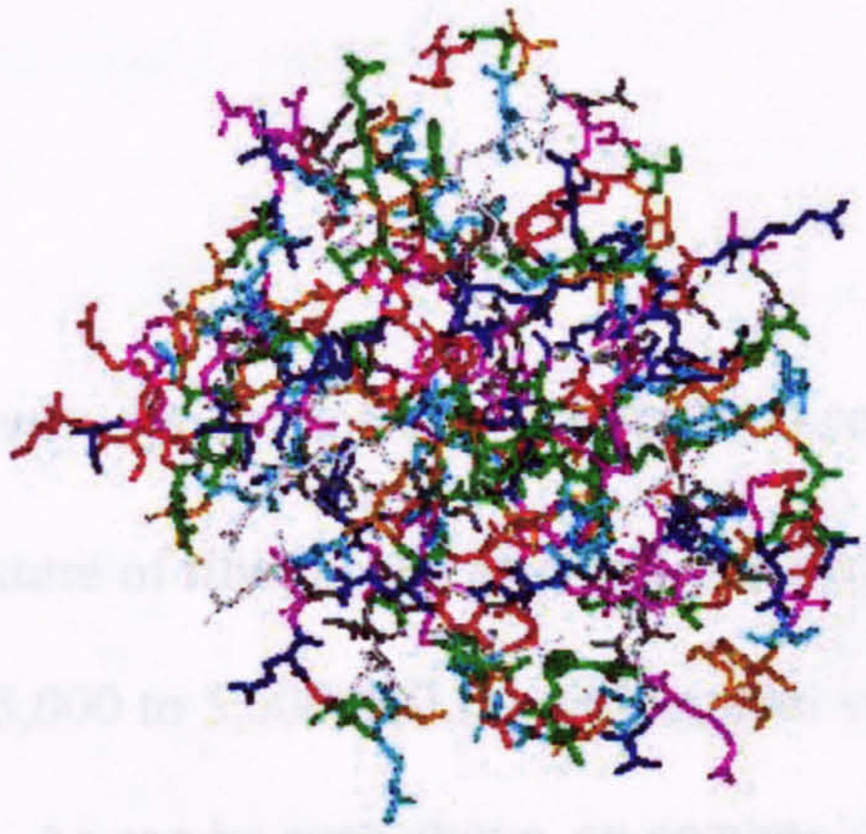


Figure 1.1. Stick representation of the serine protease, elastase.[2]

It was not until the 19th century that scientists addressed the question of whether the entity responsible was a living species or a chemical substance. In 1897 Eduard Buchner observed that extracts of yeast, containing no living cells, were able to carry out the fermentation of sugar to alcohol and carbon dioxide. He

proposed that a species called 'zymase' found in yeast cells was responsible for fermentation. In 1926 James Sumner crystallised the enzyme urease from Jack Beans and proved beyond doubt that biological catalysis was carried out by a chemical substance. His investigations showed the crystalline urease structure was overwhelmingly protein and attributed this to being the enzyme. However, he was met with much scepticism whereby other investigators argued the enzymes were not necessarily the protein but other small molecules trapped within the crystal structure. Finally, about ten years later, Sumner's conclusions gained full acceptance through further work and elaborate experiments conducted by Moses Kunitz and John Northrop and the remarkable catalytic force of enzymes was credited to chemical molecules called proteins.

1.1.2 Structure

Enzymes are large polypeptide molecules (with the exception of ribosomes which contain a mixture of ribonucleic acid and protein)[3] whose molecular weight varies from 5,000 to 5,000,000 Da with typical values in the range of 20,000-100,000 Da. As can be seen above, an enzyme's overall geometrical structure is complex. However, this can be simplified into four main substructures: primary, secondary, tertiary and quaternary.

a) A polypeptide chain consists of a series of α-amino acid building blocks joined together *via* amide bonds to form a linear sequence. This is the primary structure of the polypeptide (Figure 1.1).

b) There are at least three common stable forms of secondary structure commonly observed in proteins: the α-helix, the β-sheet and the β-turn, which are all controlled by hydrogen bonding interactions available. Hydrogen bonding between the carbonyl oxygen of one amide linkage and the N-H of the linkage four residues ahead of the chain within a single polypeptide gives rise to an α-helix (Figure 1.2). This results in all the amino acid side chains pointing outwards from the pitch of the helix. The β-sheet is a structure formed by two or more linear polypeptide strands, held together by a series of interstrand hydrogen bonds. There are two types of β-sheet structures: parallel β-sheets, in which the peptide strands both proceed in the same amino-to-carboxyl direction; and antiparallel, in which the peptide strands proceed in opposite directions. The β-turn is a structure often formed at the end of a β-sheet which leads to a 180° turn in the direction of the peptide chain.

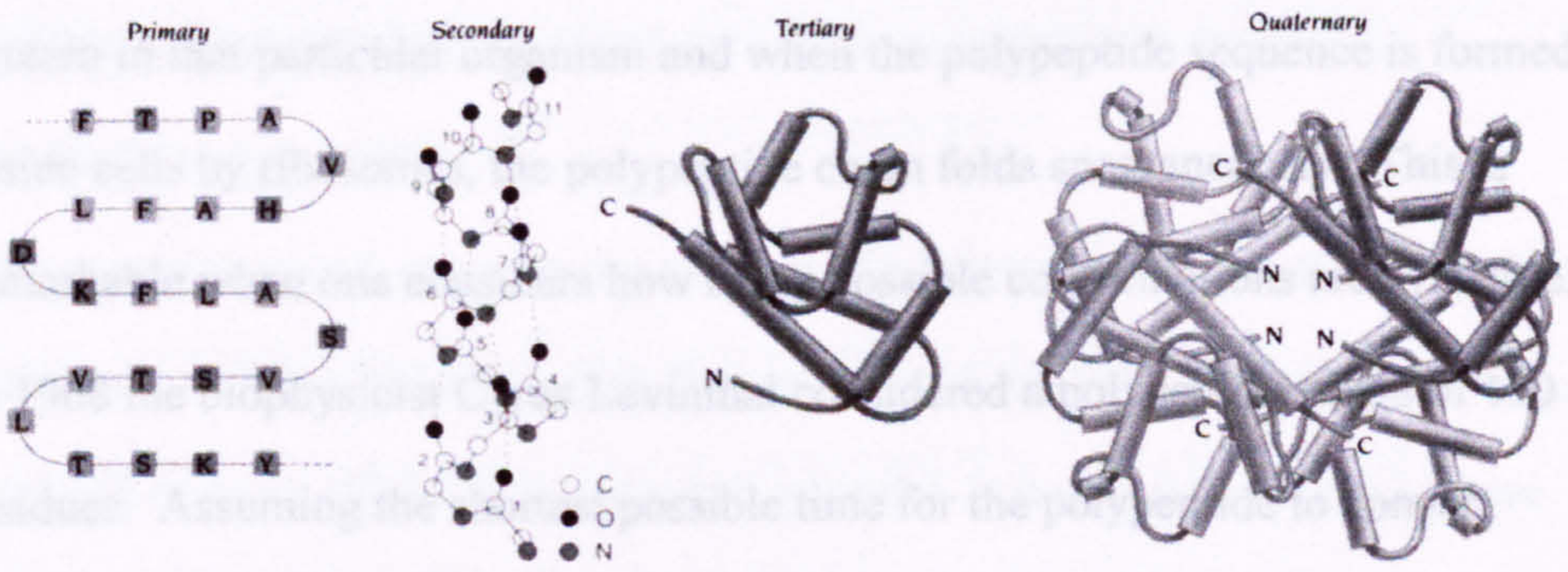


Figure 1.2. The four basic structures of enzymes.[4]

c) The three-dimensional structure of protein sub-units, known as the tertiary structure, arises from combining elements of secondary structure to form one or several compact globular units called domains. The packing of secondary

structural units usually involves burying hydrophobic amino acid side chains on the inside of the protein and positioning hydrophilic amino acid side chains on the surface.

d) Frequently, proteins consist of a number of domains where a particular domain has a specific function, such as binding a substrate or cofactor. Larger proteins often consist of more than one tertiary structure, which fit together to form the active quaternary structure.[5]

The process which produces the secondary, tertiary and the unique overall stable and active geometry of an enzyme, is referred to as folding. The sequence of amino acids in the polypeptide chain is all-important for this to occur and contains all the information needed. In living organisms the amino acid sequence is controlled by the nucleotide sequence of the corresponding gene, i.e. the piece of deoxyribonucleic acid (DNA) which encodes for that particular protein in that particular organism and when the polypeptide sequence is formed inside cells by ribosomes, the polypeptide chain folds spontaneously. This is remarkable when one considers how many possible conformations are available. In 1968 the biophysicist Cyrus Levinthal considered a polypeptide chain of 150 residues. Assuming the shortest possible time for the polypeptide to convert from one conformation to another was one picosecond and simplifying the argument by only allowing three possible conformations for each peptide group. The polypeptide would have 3^{150} possible conformations to process, which would take 10^{48} years. Remarkably, the actual folding time can be between 0.1 and 1000 seconds.[4] It was also shown by Anfinsen and his colleagues at the National Institutes of Health, that it was possible to refold a particular digestive

enzyme after it had been severely denatured. They subjected the denatured enzyme to near physiological conditions of pH, salt concentration and temperature and within hours the polypeptide chain refolded, regaining full biological activity. This indeed indicates the polypeptide sequence was capable of containing all the information required to induce folding into an architecturally complex and biologically active geometry.

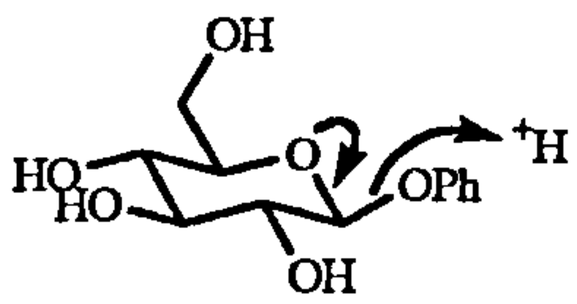
1.1.3 Selectivity and Catalysis

Enzymes are almost always proteins (the exception being ribosomes) but not all proteins are enzymes, the difference being that enzymes possess catalytic activity. The part of the enzyme's tertiary structure that is responsible for the activity is called the 'active site' and the function of enzymes is to catalyse biochemical reactions where parameters under consideration are speed, selectivity and specificity.

With regard to speed, enzymes are capable of catalysing reactions at rates well in excess of a million-fold faster than the uncatalysed reaction. Ratios of K_{cat}/K_{uncat} can be as large as 10^6-10^{14}. An illustration of this is shown in Figure 1.3 where glycoside hydrolysis is considered. In this instance, the rate of intramolecular acid-catalysed glycoside hydrolysis is 10^3 fold faster than acid-catalysed glycoside hydrolysis. However, the enzyme-catalysed glycoside hydrolysis is even faster than acid-catalysed glycoside hydrolysis, by 10^7 fold.

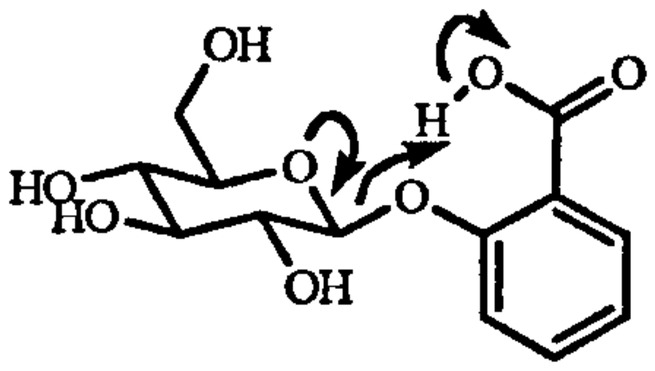
An example of an extremely efficient enzyme is acetylcholinerase, which has a turnover number of 25,000 reactions catalysed per second, meaning that it

cleaves an acetylcholine molecule in just 40 millionths of a second. This is incredibly fast and the reaction involved begins to be controlled by the rate at which a substrate can diffuse onto the enzyme's active site, and the product can diffuse away into solution; the so called *diffusion-limit.*



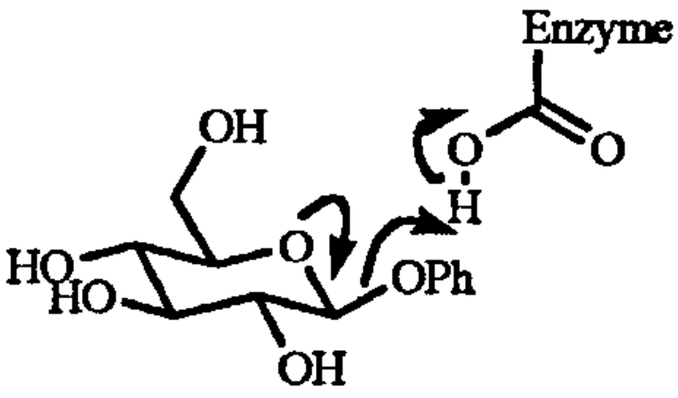
Acid-catalysed hydrolysis

$k_{obs} = 1.9 \times 10^{-6}\ s^{-1}$ in 0.1 M HCl



Intramolecular hydrolysis

$k_{uni} = 1.4 \times 10^{-3}\ s^{-1}$



β-galactosidase hydrolysis

$k_{cat} = 40\ s^{-1}$

Figure 1.3. Comparing the rates of glycoside hydrolysis *via* acid, intramolecular and enzymatic mechanisms.

As fast as the substrate can diffuse onto the active site it is processed by the enzyme before the next molecule of substrate appears. The efficiency of acetylcholine is truly remarkable and not necessary surprisingly, for it is involved in the propagation of nerve impulses at synaptic junctions: a process for which the utmost speed is needed.

Enzymes are also highly selective in the reactions that they catalyse and they need to be. Typically a biological cell may contain several hundred different chemical compounds, however the enzyme is required to *selectively* process only one of these and *specifically* carry out only one reaction pathway upon this.

1.1.4 Transition State Stabilisation

A crucial property of a catalyst is its ability to reduce the activation energy of a reaction through stabilisation of its transition state. Enzymes do the same although the situation is somewhat more complicated since there are usually several transition states in an enzymatic transformation. Another aspect is the enzymes ability to bind to the substrate. This can be viewed as aiding the reaction by holding the substrate in the enzyme's 'active site', encouraging the formation of the transition state to occur, before the substrate diffuses away again. However, it is not actually advantageous for the enzyme to bind its substrate too tightly. Tim Bugg has rationalised this in an interesting manner,[3] by examining the free energy curves for a hypothetical enzyme-catalysed reaction proceeding *via* a single rate determining transition state, shown in Figure 1.4.

Assuming it was possible to alter the enzyme E in a manner that it bound the substrate S or the transition state more tightly, and in each case the starting free energy of (E + S) was the same. In the presence of high substrate concentrations the enzyme would in practice be fully saturated, so the activation energy for the reaction would be governed by the energy difference between the ES complex and the transition state. In Fig. 1.4b the enzyme is able to bind both the substrate and the transition state more tightly (and hence lower their free energy equally). This, however, leads to no change in the activation energy, and hence no change in the rate acceleration. In Fig. 1.4c the enzyme binds only the substrate more tightly which generates a 'thermodynamic pit' for the ES complex and hence increases the activation energy, so the reaction is slower. However, if, as in Fig.

1.4d the enzyme can selectively bind the transition state, then it can reduce the activation energy and hence increase the reaction rate.

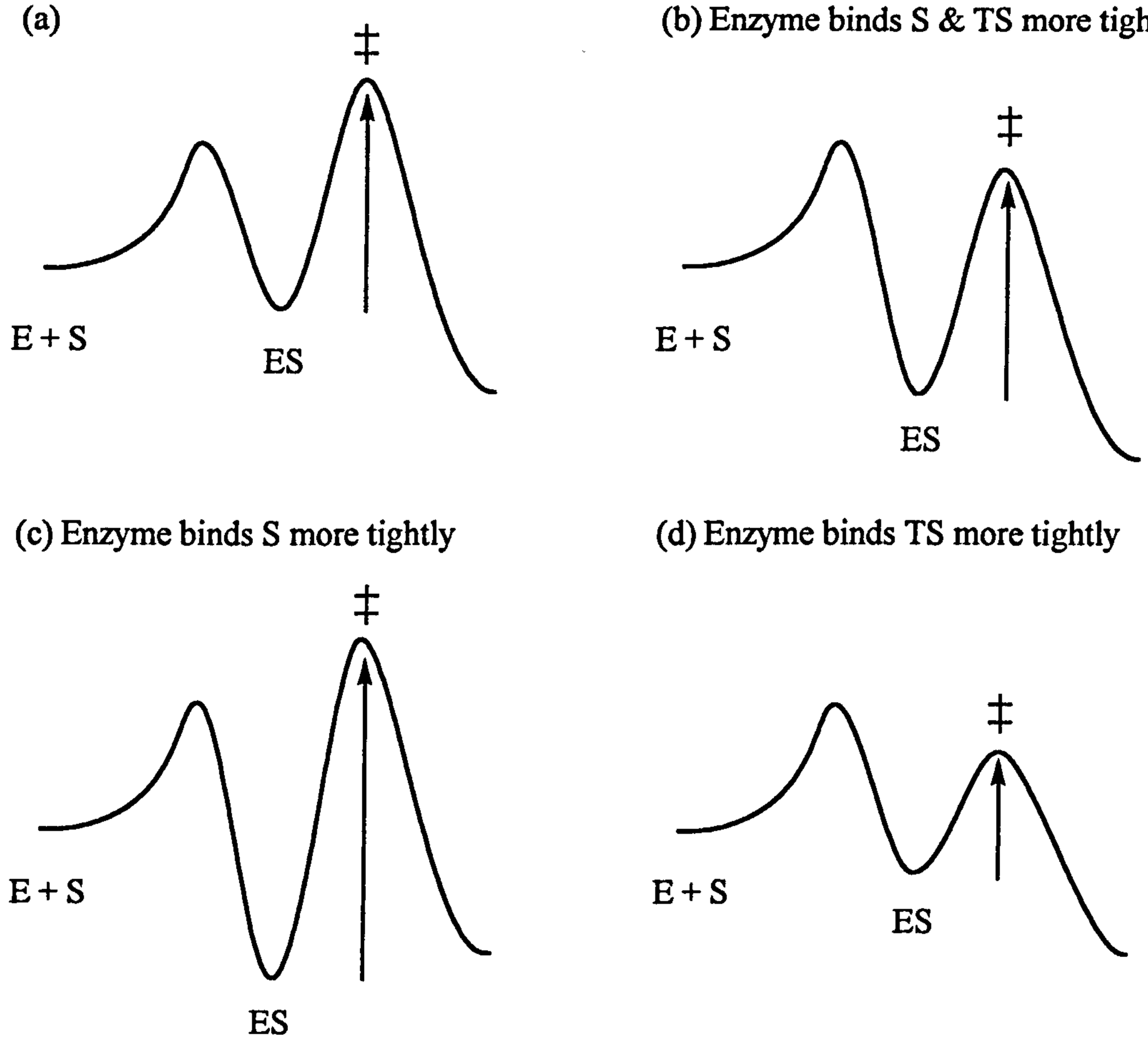


Figure 1.4. Transition state stabilisation and substrate binding.

The conclusion of this analysis is that in order to achieve optimal catalysis, enzymes are required to selectively bind the transition state, rather than the substrate and it can be clearly seen it is not advantageous for enzymes to bind their substrates too tightly.

1.1.5 Enzyme Mimics

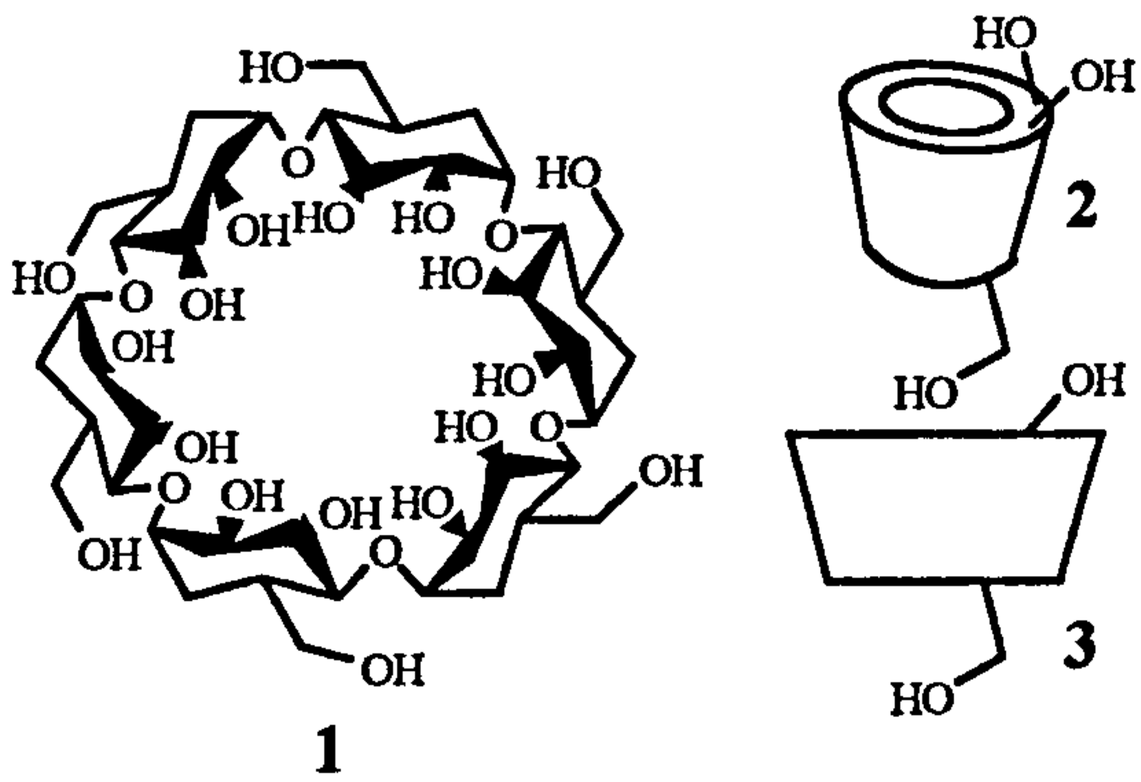
For an excellent source of information discussing enzyme mechanisms, models and mimics please refer to Anthony Kirby's review.[6] The following is a brief description to discuss some of the properties an enzyme mimic requires to be justified, followed by a very brief description of some systems that have been investigated.

Enzyme mimics catalyse reactions by mechanisms that are demonstrably enzyme-like. The minimum requirement is that the reactions concerned should involve an initial binding interaction between the substrate and the catalyst. This gives rise to Michaelis-Menten kinetics and the reactivity is measured in terms of the familiar parameters k_{cat} and K_M. Real enzymes are more than just highly evolved catalysts: they also recognise and respond to molecules other than their specific substrate and product, as part of the control mechanism of the cell. The evolution of artificial enzymes is at a more primitive stage, with efficient catalysis the primary objective. Systems are known that model various other functions, such as potential control mechanisms. However, to be useful as an industrial catalyst, for example, an artificial enzyme does not need sophisticated built-in feedback control or high substrate specificity

A major problem encountered by enzyme mimics studied to date has proven to be product inhibition where the transition state has been stabilised but so too has the product. This inevitably leads to product inhibition and a loss of product turnover. The following are some examples of enzyme mimic systems.

1.1.5.1 Cyclodextrins

Cyclodextrins consist of a hydrophobic cavity lined with CH groups and glycosidic oxygen atoms, and carry an array of hydroxyl groups on both rims (Scheme 1.1). The full structural formula **1** can be represented in the simpler form **2**, often showing only the single functional group of interest **3**.

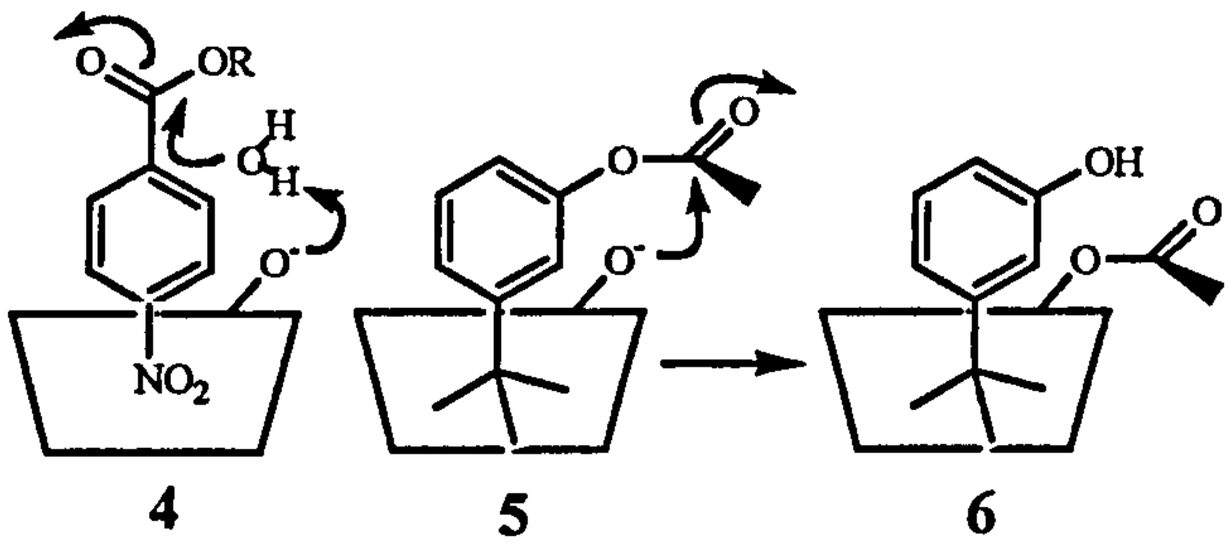


Scheme 1.1. Schematic representation of cyclodextrins.

As can be seen the structure is slightly more open on the side of the secondary OH groups. This, plus some steric hindrance by the primary 6'-CH_2OH groups on the other face, makes the face more accessible to hydrophobic guests which can bind in the cavity if they are of the right size.[6]

Unmodified cyclodextrins are themselves good functional enzyme mimics (Scheme 1.2) where their secondary OH groups, with pKa values in the range 12-12.5, can be efficiently involved in nucleophilic attack towards, for example, nitrophenyl esters. The nucleophilic reaction is inefficient for *p*-substituted benzene derivatives **4** due to the ester group being held in the central axis of the cyclodextrin upon tight binding of the substrate. This holds the ester group too

far from the secondary OH groups, however, if general base catalysis begins to compete, a true catalytic system is realised. i.e. The catalytic group is unchanged and immediately available for further reaction. The nucleophilic mechanism on the other hand, which is favoured for aryl acetates with substituents in the meta position **5** of the aromatic ring, gives the acylated cyclodextrin **6** as the initial product. However, quite often the intermediate ester is more stable than the original substrate and the subsequent hydrolysis becomes rate limiting. Product inhibition in not over come as the product is still bound. The problem is typically resolved by using an excess of cyclodextrin but then the observed 'catalysis' does not involve turnover.

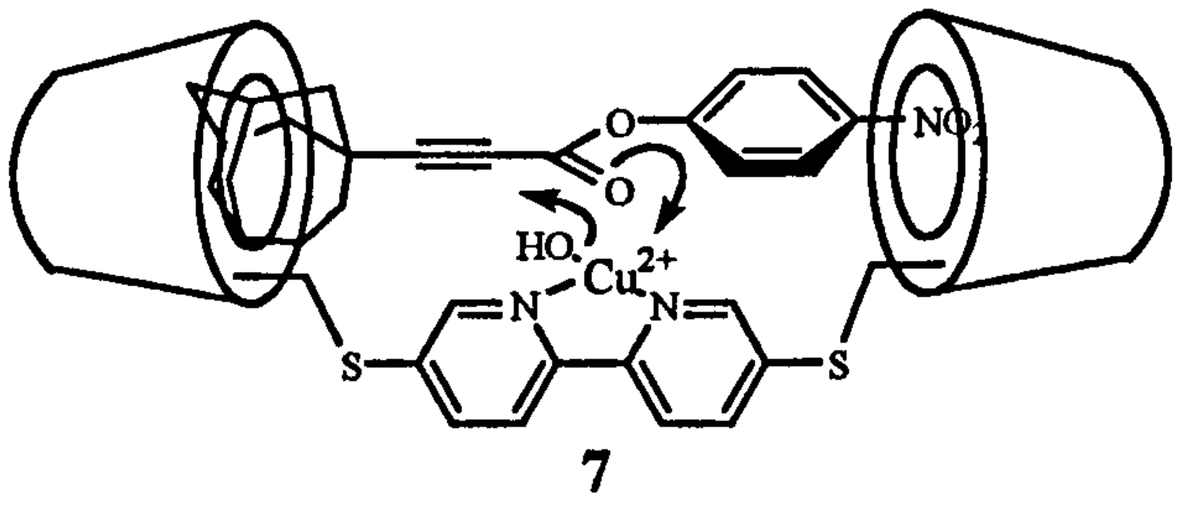


4 **5** **6**

Scheme 1.2. Unmodified cyclodextrins.

Cyclodextrin catalysis can be improved by modifying the OH functional groups, for example with imidazole.[7] The substrate can also be modified through the use of ferrocene to efficiently bind the cavity.[8-10] Another interesting approach to improving the cyclodextrin system is to form dimers through a covalent linkage **7** which is itself included in the catalytic cycle (Scheme 1.3). The Cu^{2+} complex is an efficient catalyst for the hydrolysis of the adamantanyl ester which was problematic for the cyclodextrin monomer because the ester was bound in

the wrong orientation. This time the system achieves turnover because the bifunctional binding of the substrate is sufficiently stronger than the two halves produced upon the intramolecular ester cleavage, so that product inhibition is avoided.



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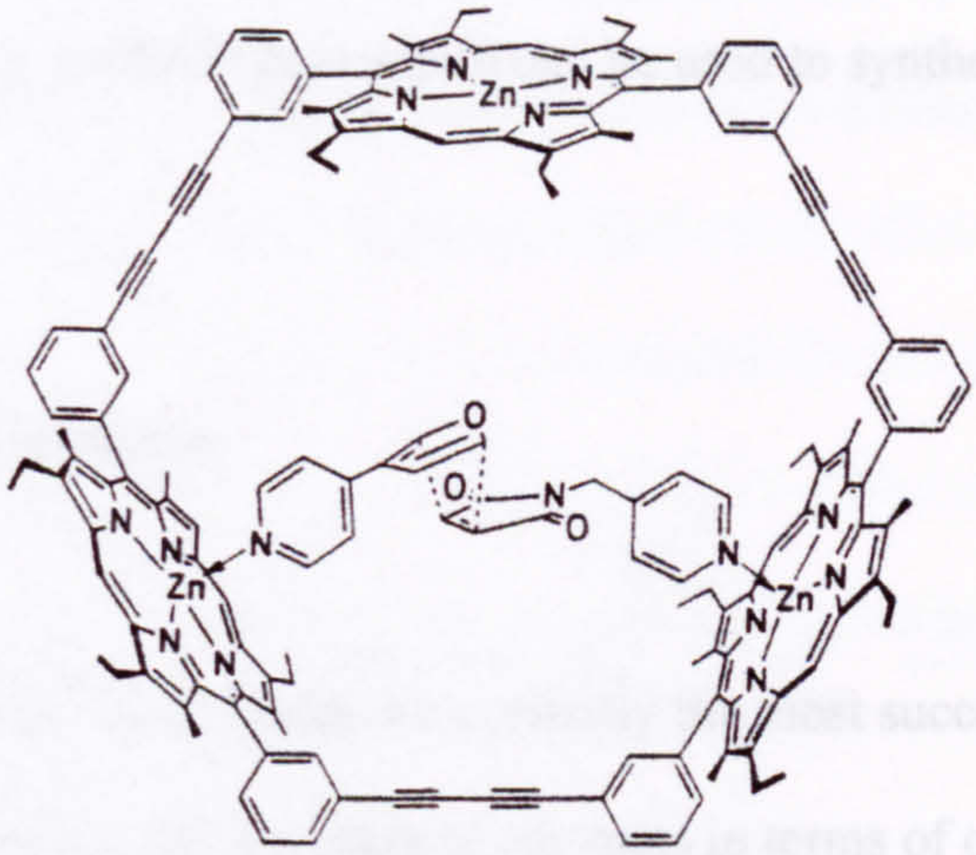
Scheme 1.3. Cyclodextrin dimer tethered together *via* a covalent linkage.

1.1.5.2 Synthetic Hosts

The previous section was concerned with the use of a model to effect the cleavage of a particular bond. Another area of interest is designing systems which will a) bring two substrates into close proximity which allows them to react with each other (and would ideally catalyse this reaction too), b) bind single substrates and then react with them.

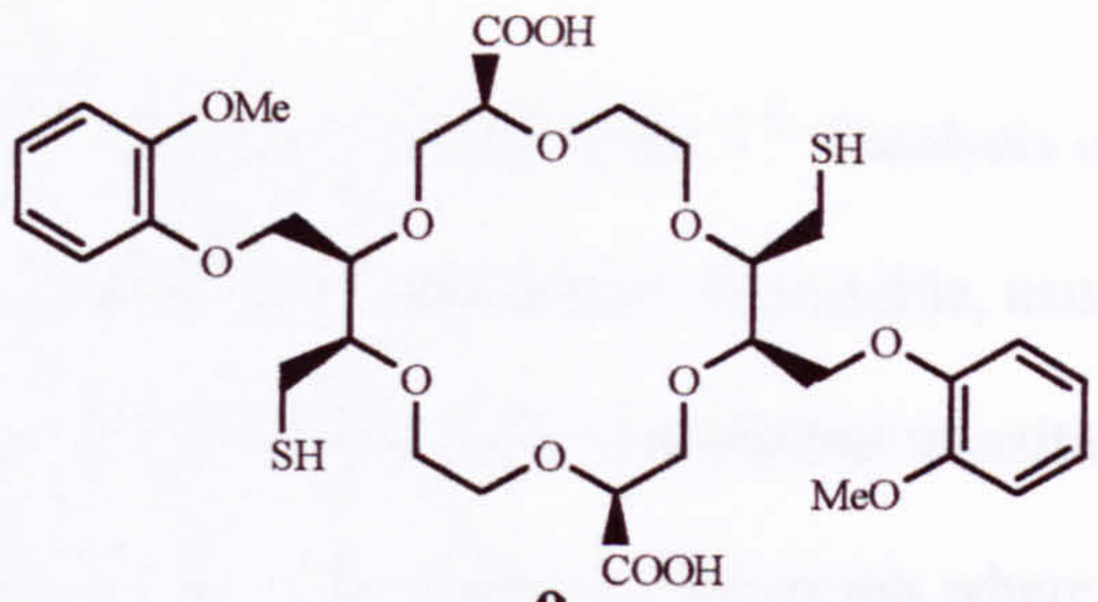
a) An example of a synthetic host behaving as a matchmaker is that of the porphyrin host **8** (Scheme 1.4). The porphyrin trimer binds symmetrical di- and tri-pyridyl systems very strongly and the substituted monomeric pyridines well enough to accelerate the Diels-Alder reaction between suitable guests when they are bound inside the cavity. The binding also controls the stereochemistry of the

reaction well enough to reverse the normal kinetic selectivity, so that the *exo* adduct is produced up to 1000 times faster than the corresponding *endo* isomer. Unfortunately the product also binds strongly, eliminating turnover and hence catalysis.



Scheme 1.4. Porphyrin synthetic host **8**.

b) An example of a synthetic host used to form a new covalent bond after first binding and reacting with the substrate itself is **9** (Scheme 1.5). Devised by Koga *et al.*,[11] (which is a simpler dithiol that can be used to synthesise peptide bonds)[12] this was based on earlier work which had shown that crown ethers bind the ammonium group of amino acid derivatives, and that bound activated esters will acylate groups like SH attached to the crown. Loading **9** with first one and then a second α-amino acid (as thioesters) allows intramolecular aminolysis and the formation of the dipeptide, as the thioester.



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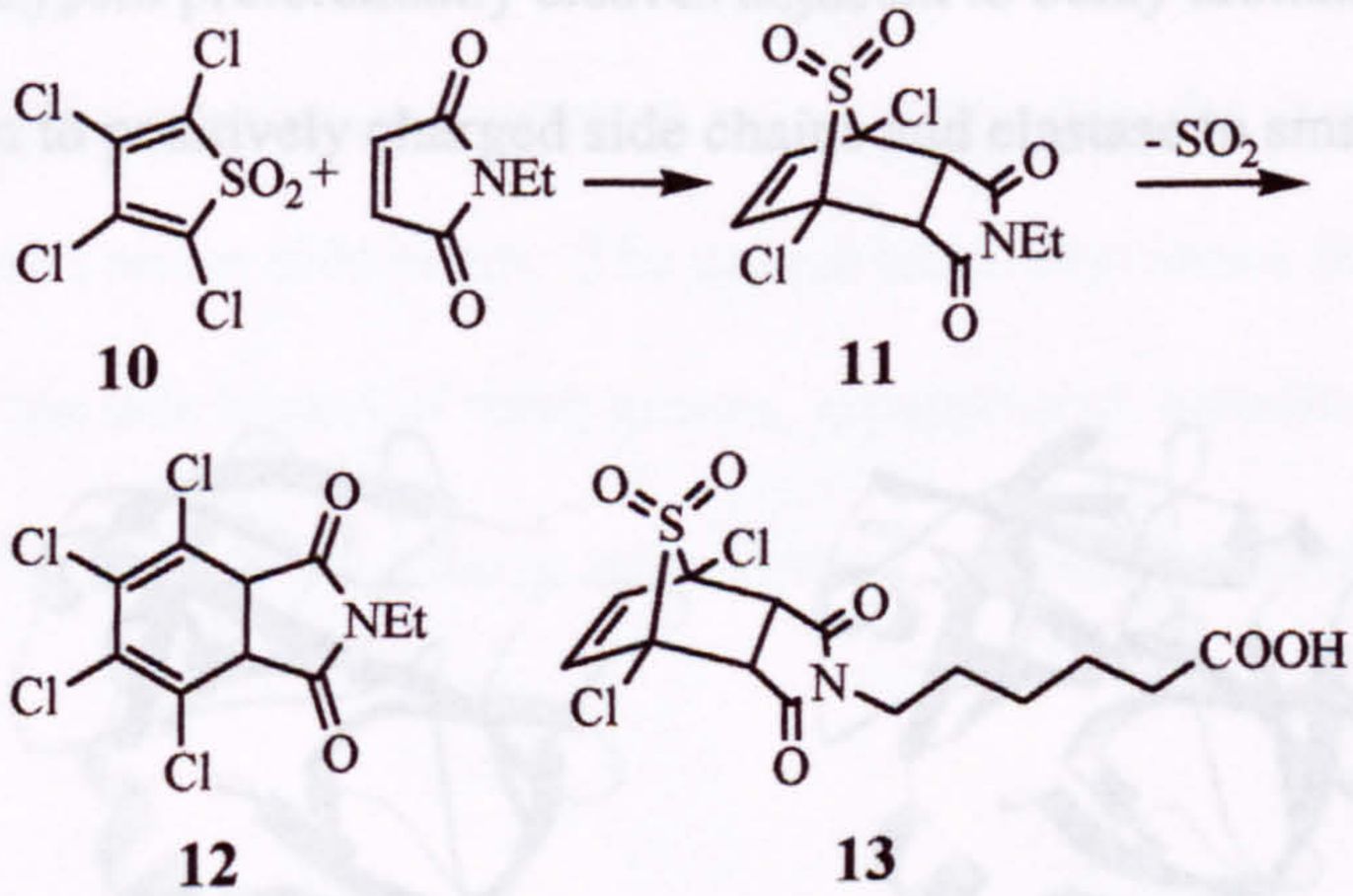
Scheme 1.5. A synthetic host which can be used to synthesise dipeptides.

1.1.5.3 Catalytic Antibodies

Catalytic antibodies[13] or abzymes are currently the most successful enzyme mimics, though they too fall far short of enzymes in terms of catalytic efficiency. They are proteins raised in a biological system specifically against transition state analogues (haptens) for the reaction of interest. Consequently, the problems of design and synthesis involve in principle only small molecules on a scale familiar to the organic chemist. Under the right conditions the immune system responds to the transition state analogue by producing vast numbers of antibodies that recognise and bind to it - literally an exercise in the molecular recognition of transition states. The primary limitations are the accuracy of the hapten as a model for the transition state and the effectiveness of screening the large numbers of antibodies produced for catalysis.

Though their specificity and potential regio- and enantioselectivities are good, current catalytic antibodies are not much more efficient than other enzyme mimics. They have proved quite good at catalysing certain reactions, like the hydrolysis of *p*-nitrophenyl esters and have been reported to catalyse the

hydrolysis of benzyl[14] and other alkyl esters.[15] Catalysis of the hydrolysis of activated esters is rather reliably obtained with suitable, usually phosphonate haptens which model the structure of the tetrahedral transition states involved. Again this methodology can suffer product inhibition where by the product binds to the antibody after formation and thus inhibits turnover. A strategy against this is to design the reaction so that the product structure is distinctly geometrically different from its transition state (Scheme 1.6).



Scheme 1.6. An approach of using a hapten to generate a catalytic antibody.

Thus Hilvert's antibody catalysing the Diels-Alder reaction of tetrachlorothiophene dioxide **10** and N-ethylmaleimide achieves turnover because the initial adduct **11** rapidly loses SO_2 to give the bicyclic product **12**.[16] Catalysis results simply from productive binding. The hapten **13** is a reasonable transition state analogue but geometrically very different from the final product.

1.1.6 Serine Proteases

Serine proteases are a class defined by the presence of a uniquely reactive serine side chain.[5,17] They hydrolyse the same type of substrate, namely polypeptide chains of proteins, however, different members of the family preferentially cleave polypeptide chains at sites adjacent to different amino acid residues. This is illustrated by considering the enzymes chymotrypsin, trypsin and elastase who have very similar three dimensional structures but different specificity (Figure 1.5). Chymotrypsin preferentially cleaves adjacent to bulky aromatic side chains, trypsin to positively charged side chains and elastase to small uncharged side chains.

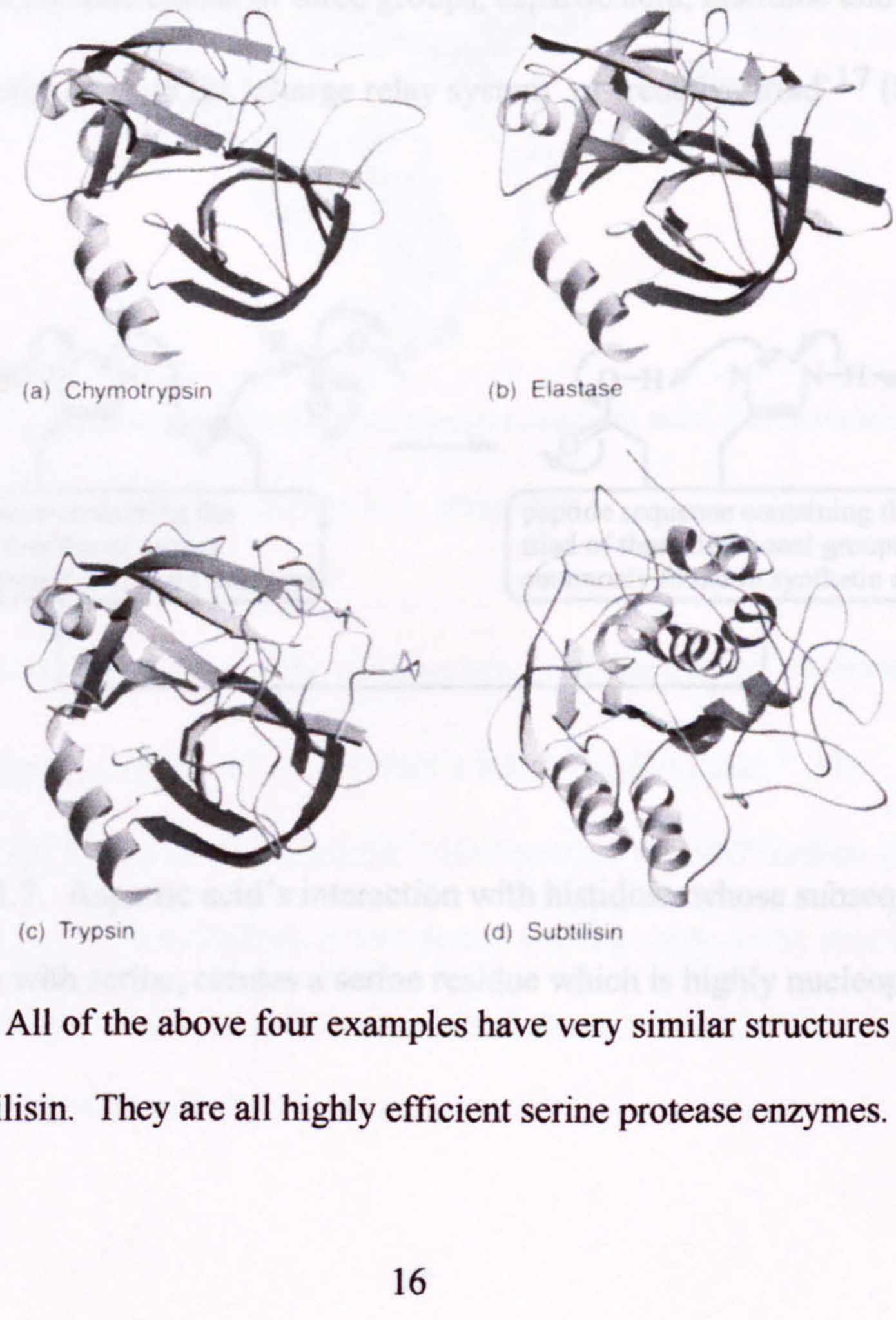
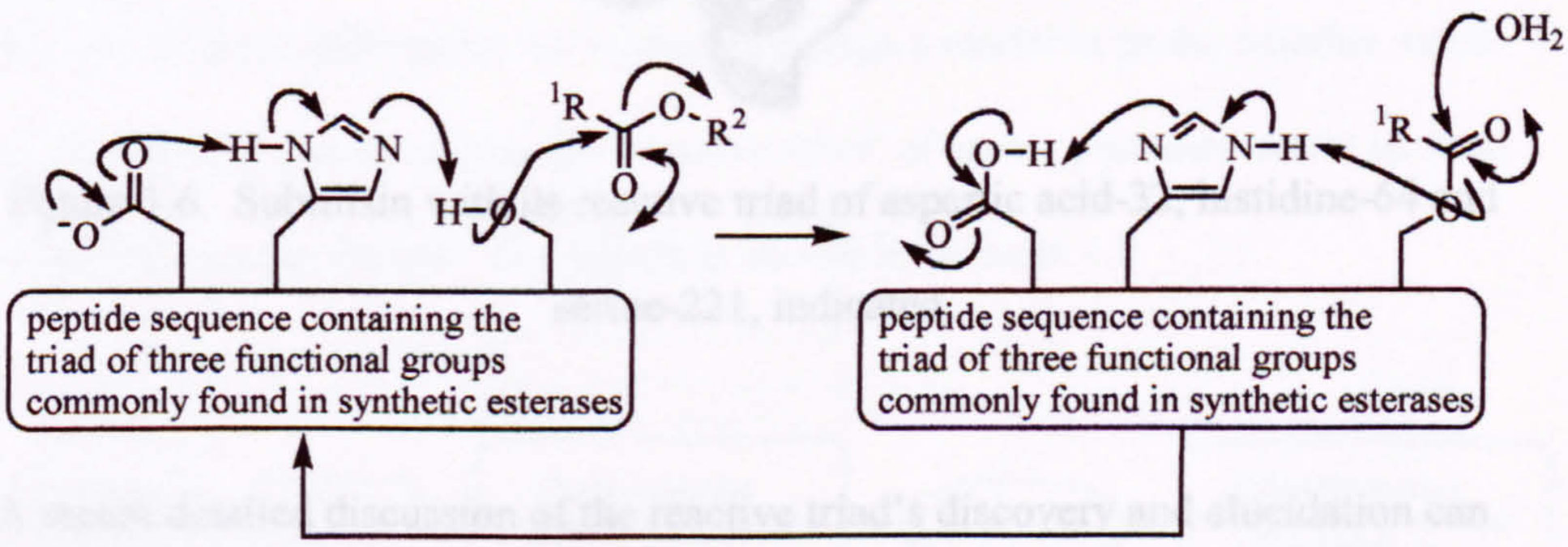


Figure 1.5. All of the above four examples have very similar structures except subtilisin. They are all highly efficient serine protease enzymes.

Subtilisin is another serine protease and despite having a completely different three dimensional structure to the examples above, its active site is remarkably similar. Interestingly, much of our understanding of basic enzymology comes from studies on the serine proteases, notably on chymotrypsin and trypsin, because of their ready isolation in large quantities from pancreases from slaughter houses. However, subtilisin been pushed to the fore of investigation owing to its lack of disulfide crosslinks, meaning its handling is far easier, and its commercial use in soap powders has meant it has been readily available. It is obtainable in high yields from E. *coli* allowing production on the ton scale.

As mentioned above serine proteases are a class defined by the presence of a uniquely reactive serine side chain. The unique reactivity comes from the interaction of the side chains of three groups, aspartic acid, histidine and serine commonly referred to as the 'charge relay system' or 'reactive triad'[17] (Scheme 1.7).



Scheme 1.7. Aspartic acid's interaction with histidine, whose subsequent interaction with serine, creates a serine residue which is highly nucleophilic.

For subtilisin these refer to the units 32, 64 and 221 respectively (Figure 1.6). They are held in a favourable spatial configuration and orientation by the rest of the enzyme's massive structural architecture, resulting in the activation and increase in nucleophilic strength of the serine hydroxy side chain by the negative charge on the carboxylate *via* the histidine. Hydrolysis of the serine substrate ester bond by water regenerates the enzyme and the hydrolysed substrate.

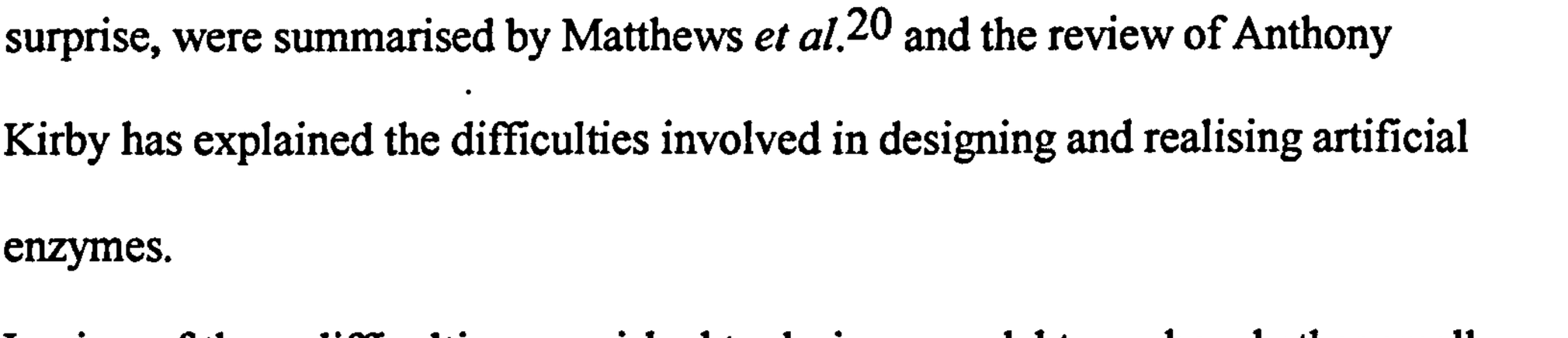
Figure 1.6. Subtilisin with its reactive triad of aspartic acid-32, histidine-64 and serine-221, indicated.

A recent detailed discussion of the reactive triad's discovery and elucidation can be found in chapter sixteen of Alan Fersht's latest excellent text.[5] The importance of the oxyanion binding site with regard to the stabilisation of the carbonyl-tetrahedral intermediate is mentioned and the controversy around whether the reactive triad operates as described above or whether the mechanism is justified *via* a low-barrier hydrogen bond.

1.1.7 Synthetic Esterases

An interesting paper was published in 1993 by Atassi and Manshouri[18] who claimed that an artificial enzyme system did attain catalytic efficiency that rivalled that of natural enzymes. 'Pepzymes' consisting of small 29 residue peptides, one of which had been designed by surface-simulation synthesis to mimic the active site of α-chymotrypsin and cyclised by a disulfide bond, was reported to hydrolyse the simple trypsin substrate N-tosyl-L-arginine methyl ester with catalytic efficiencies comparable to α-chymotrypsin. Not surprisingly, this claim generated a great deal of scepticism and excitement, and was later challenged by other groups.[19,20] The specific reasons as to why this was no surprise, were summarised by Matthews *et al.*[20] and the review of Anthony Kirby has explained the difficulties involved in designing and realising artificial enzymes.

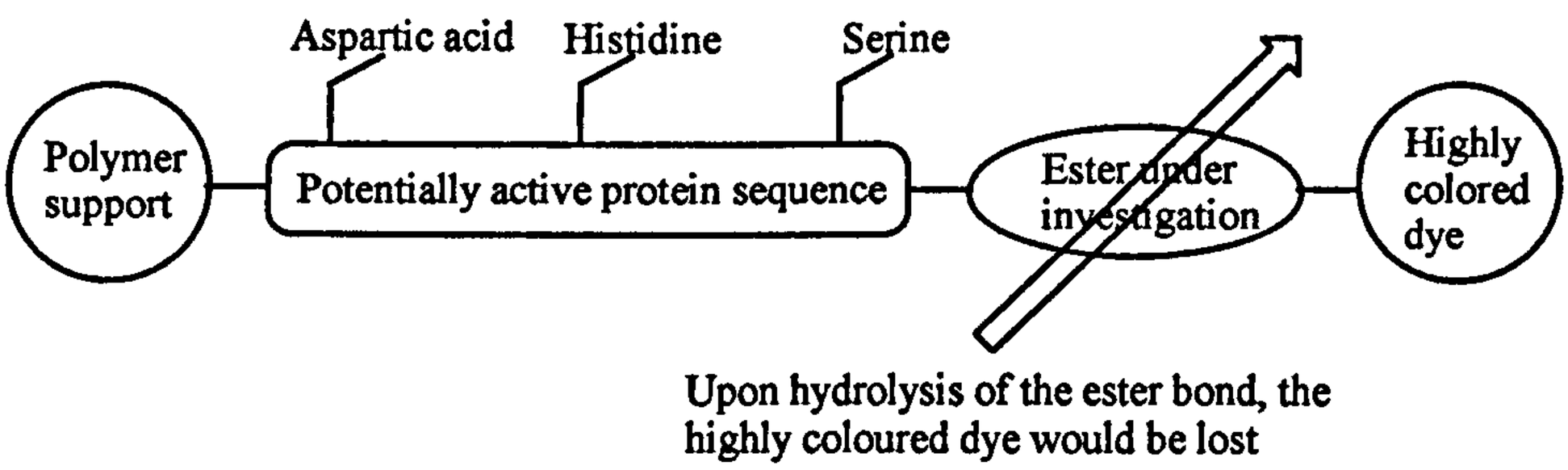
In view of these difficulties we wished to design a model to probe whether small linear peptides incorporating the 'reactive triad' of serine proteases could be used to hydrolyse ester bonds. The design is shown in Scheme 1.8.



Scheme 1.8. The reactive triad incorporated within small linear peptides.

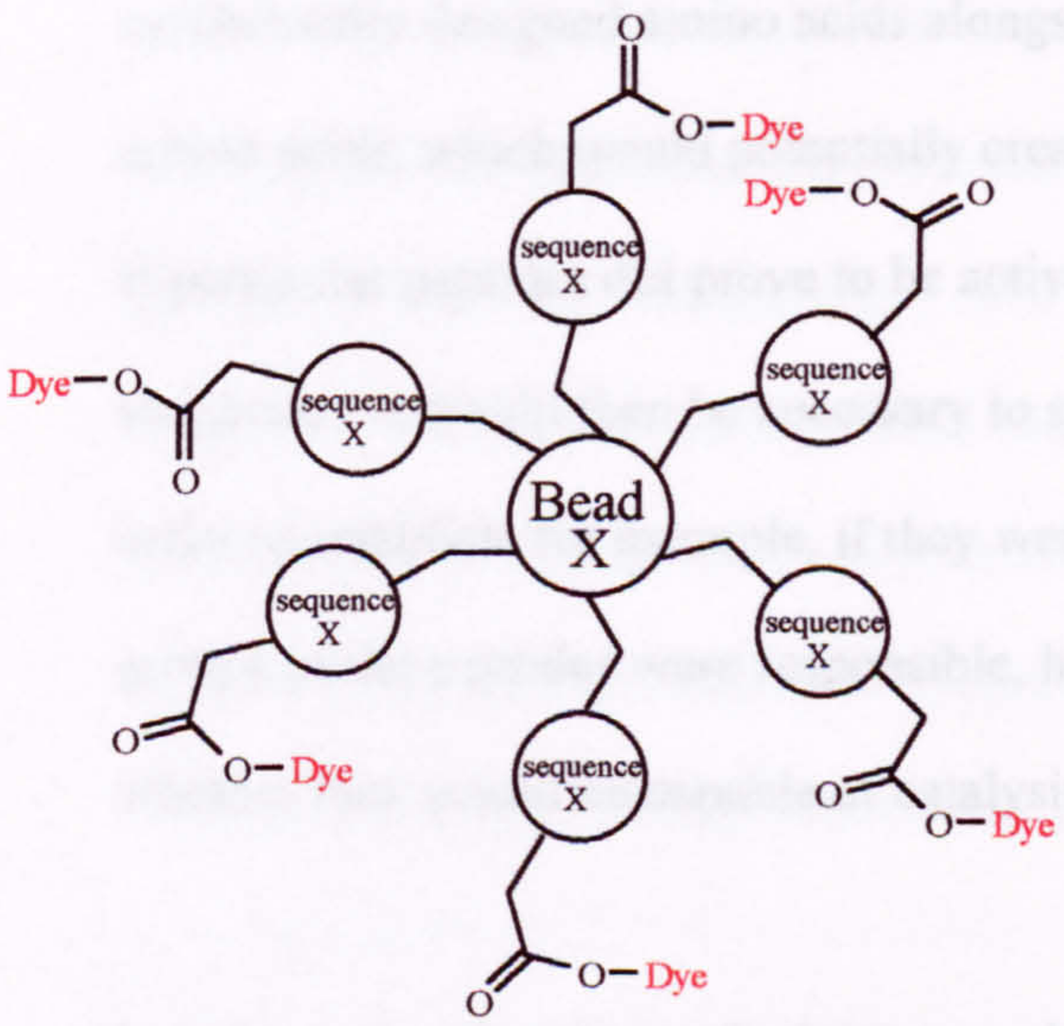
The peptides, bound on a suitable polymer support, could be synthesised so that each of the main components of the reactive triad would be present and combinatorially spaced apart with a range of other naturally occurring amino acids. This would create a combinatorial library with a large range of potentially active protein sequences. Hopefully a particular sequence would hold its reactive triad in the just the correct spatial configuration and orientation necessary to yield an effective synthetic serine esterase.

Several problems are immediately evident in designing such a system even if a particular sequence were to show the activity desired. One of which includes the elucidation of active sequences. This was taken into consideration by deciding to rely on intramolecular cleavage of the ester concerned, whereby one side of the ester would be tethered to the terminal end of the peptide and the other covalently linked to a dye [such as the highly active red azo dye, disperse red], (Scheme 1.9).

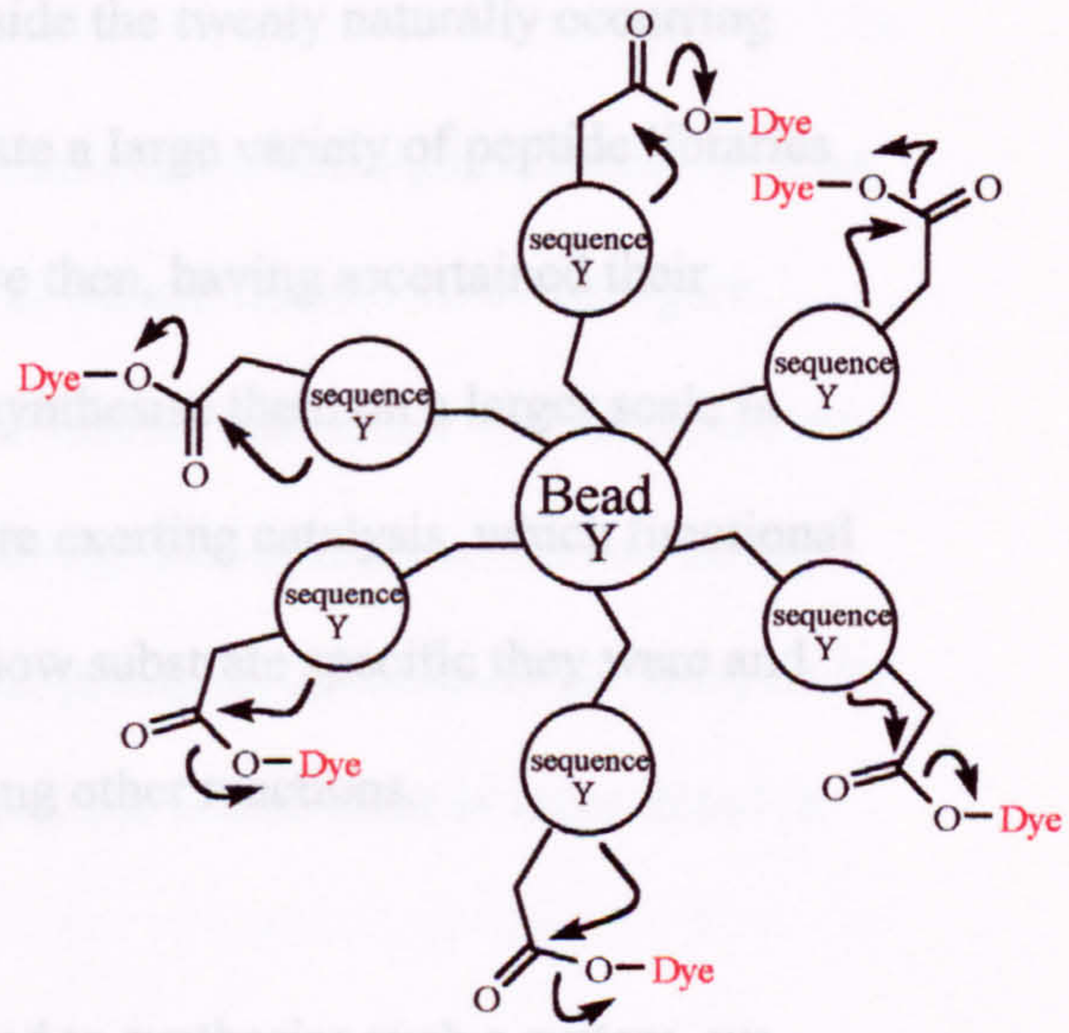


Scheme 1.9. Should a peptide sequence show activity, then the ester would be cleaved *via* intramolecular hydrolysis, and the highly coloured dye would be released.

This method is again shown in Scheme 1.10. Under the combinatorial conditions, each individual bead would be loaded with only one particular peptide sequence. The peptides would be tethered to the ester through a linker, the ester would be covalently linked to the dye and all the sequences available would be represented several times throughout the bead mixture. Therefore, upon analysing a mixture of the coloured beads under various conditions, a sequence showing activity would cleave its ester bond *via* intramolecular hydrolysis, giving a distinct colour loss. The most active sequences would then be indicated by the beads showing the least amount of colour within the bead mixture.



Sequence X is inactive and no colourloss is observed



Sequence Y is highly active and total colourloss is observed

Scheme 1.10. Within the bead mixture a single bead would only support one particular sequence. Only active sequences would show colour loss.

In this situation a labile linker would need to be incorporated between the support and peptide sequence. The decoloured beads could then be hand picked from the bulk, their peptides cleaved from the support and the sequences deduced by Edman Degradation and mass spectrometry.

1.1.8 Summary of The Results Presented in This Thesis

Using this visual technique we envisaged it would be possible to analyse a large number of peptide systems very quickly. This would be highly desirable when changing parameters such as peptide length and incorporating the use of synthetically designed amino acids alongside the twenty naturally occurring amino acids, which would potentially create a large variety of peptide libraries. If particular peptides did prove to be active then, having ascertained their sequences, it would then be necessary to synthesise them on a larger scale in order to establish, for example, if they were exerting catalysis, which functional groups of the peptides were responsible, how substrate specific they were and whether they would be capable of catalysing other reactions.

In order to develop the methodology needed to synthesise such a system, we initially used a model utilising only one peptide sequence (Figure 1.7). This was chosen from literature precedent,[21] and the entirety of the research performed to date, has been on this. The peptide of choice was a modified version of the sixteen unit sequence, Lys-Ala-Ala-Ala-Glu-His-Lys-Ala-Ala-His-Glu-Leu-Glu-Asp-Pro-Asp-NH. Jacqueline Barton previously developed a system where the

above peptide was covalently linked to a rhodium intercalator, which had been shown to bind DNA. The peptide had been designed to have a high degree of α-helicity within its structure by incorporating a glutamate – lysine salt bridge and use of space filling alanines. The two histidines were assumed to create a zinc co-ordination site on one side of the α-helix and Jacqueline Barton proposed the rhodium intercalator would bring the peptide into close proximity to the DNA and effect its hydrolysis in the presence of zinc. Indeed, successful hydrolysis was observed. We decided to use the same peptide sequence but change one of the histidine residues to create the reactive triad within a similar geometrical environment. This gave the new sequence, Lys-Ala-Ala-Ala-Glu-Ser-Lys-Ala-Ala-His-Glu-Leu-Glu-Asp-Pro-Asp-NH.

The peptide was synthesised by conventional manual solid-phase methods using an Fmoc strategy on Tentagel resin, 10% of which, not shown in the figure below, was loaded with an acid labile Rink linker. The linker was incorporated to allow partial cleavage of the peptide and any subsequent derivatives during the acidic deprotection stage of the peptide, giving an opportunity for analysis by mass spectrometry. The synthesis of the peptide is discussed in more detail in the next chapter.

Another potential problem that could be envisaged for the system below was that the ester group, being hydrolysed intramolecularly, needs to reach the active serine residue. To overcome this a variety of different lengthed glycol linkers tethering the ester to the peptide were incorporated. Two different ester groups were also initially used to allow even more flexibility and as can be seen the

highly red coloured azo dye, disperse red, was covalently linked to the other side of the ester. This gave ten individual systems which we could subject to various pH ranges, co-solvents and additives to allow the optimum hydrolysis conditions to be evaluated.

An important property of enzymes is their ability to induce a high level of enantioselective control. Once our system was successfully synthesised and a variety of conditions to effect hydrolysis had been examined, we found that only one of the two esters (n = 2) afforded reasonable hydrolysis and the optimum linker length was five glycol units long. Using these parameters we decided to investigate if our system was capable of inducing any enantioselective control. This was undertaken by incorporating a racemic chiral centre on the carbon alpha to the ester carbonyl to give the system shown in Figure 1.8. Subsequent cleavage of the ester group and analysis *via* chiral HPLC was envisaged to clearly indicate if any enantioselective control was afforded during the hydrolysis. Unfortunately, the addition of the methyl group alpha to the ester's carbonyl eliminated any hydrolysis previously observed in all conditions except one, where the amount of material released was too low for analysis *via* chiral HPLC. We rationalised the lack of the new systems activity could have been due to the methyl group providing too much steric hindrance near the ester, preventing nucleophilic attack at its carbonyl. The extra steric hindrance might have also altered the geometry of the peptide as it came closer to effect the hydrolysis, causing it to become deactivated.

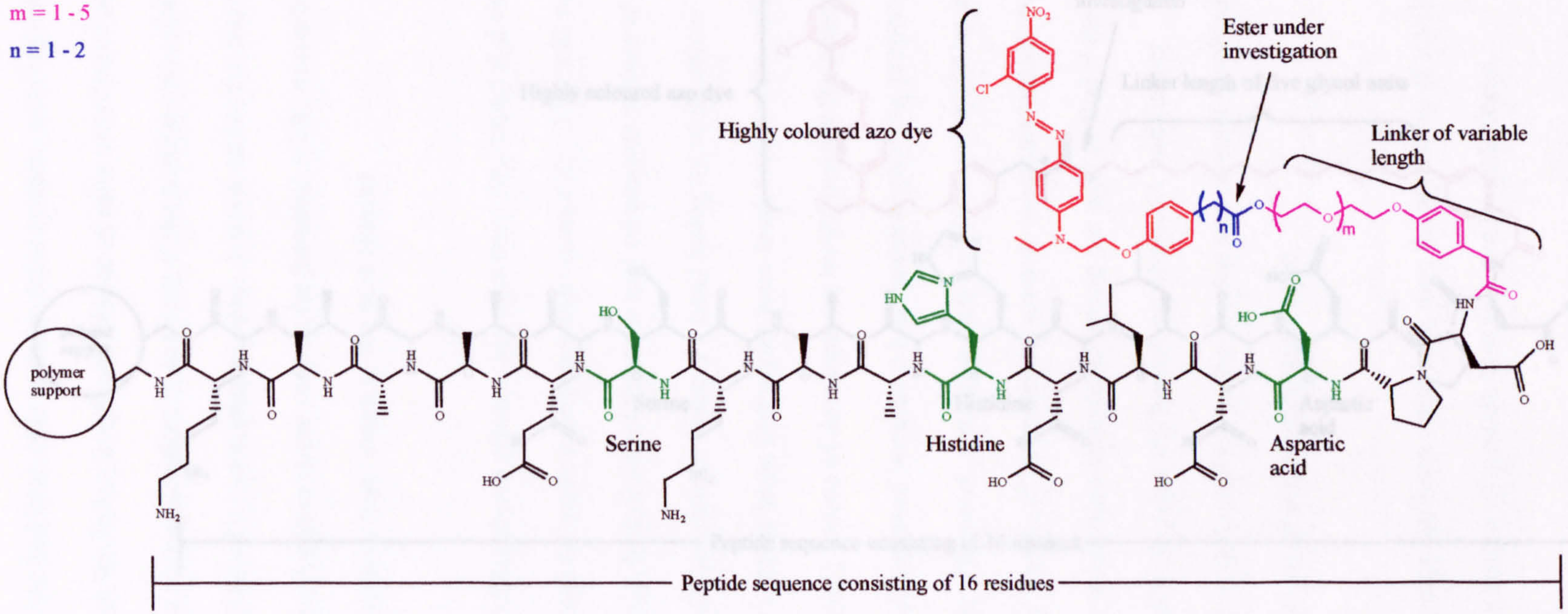


Figure 1.7. Our studies involved using only one peptide. Initially the optimum length of linker and the effects of changes near the ester, were investigated.

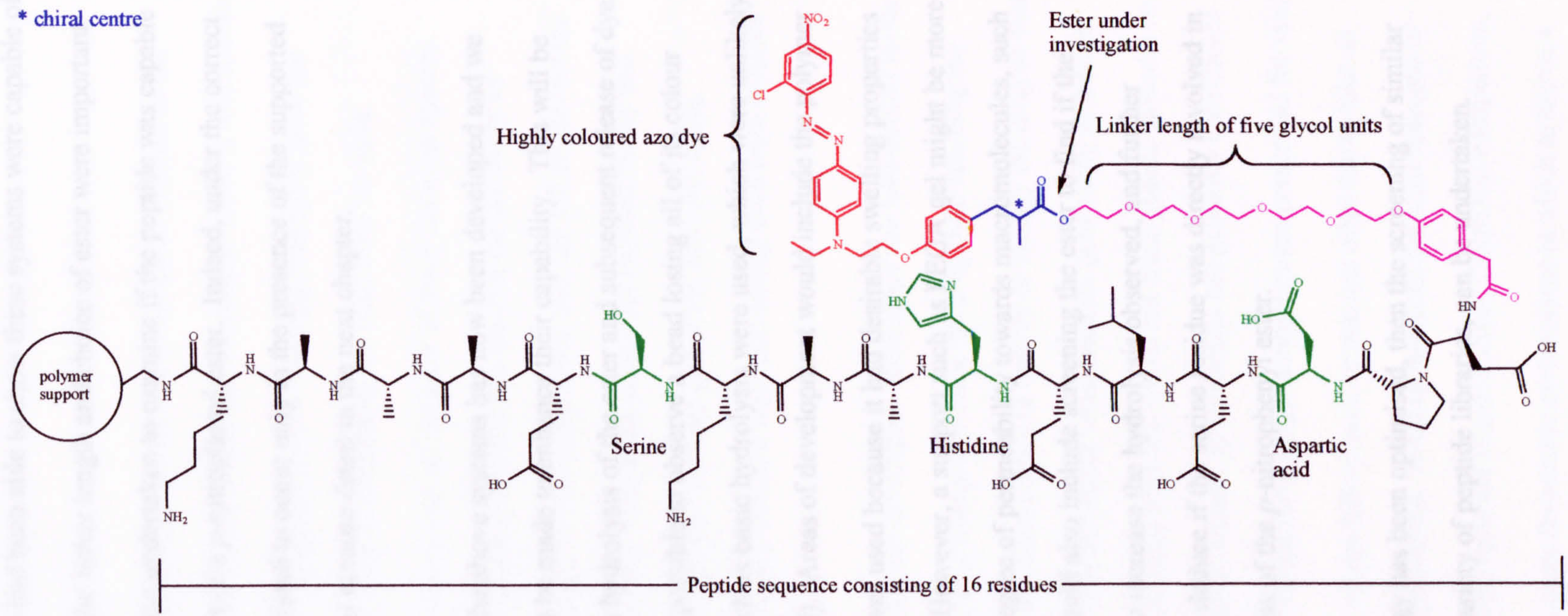


Figure 1.8. The system was modified by incorporating a chiral centre alpha to the esters carbonyl.

Although the addition of the chiral centre did remove most of the activity previously experienced, we had been able to show these systems were capable of showing hydrolysis where the linker length and choice of ester were important. A brief investigation was also undertaken to examine if the peptide was capable of intermolecular hydrolysis of a *p*-nitrophenyl ester. Indeed, under the correct conditions hydrolysis was found to occur only in the presence of the supported peptide and this is discussed in more detail in the next chapter.

The methodology to build the above systems has now been developed and we envisage improvements can be made to enhance their capability. This will be necessary because although hydrolysis of the ester and subsequent release of dye was observed, it was never possible to observe a bead losing all of its colour (unless harsh conditions such as basic hydrolysis were used, which were unlikely to involve the reactive triad). Areas of development would include the polymer support. Initially tentagel was used because it had desirable swelling properties and was readily available. However, a support such as PEGA gel might be more suitable as it has a higher degree of permeability towards macromolecules, such as peptides. Other areas would also include screening the ester to find if the structure can be modified to increase the hydrolysis observed, and further investigation undertaken to deduce if the serine residue was directly involved in the intermolecular hydrolysis of the *p*-nitrophenyl ester.

Once the initial methodology has been optimised, then the screening of similar peptides and eventually a variety of peptide libraries, can be undertaken.

1.2 Vancomycin

1.2.1 Antibiotics

The observation that the mould of *Penicillium notatum* killed cultures of the bacterium *Staphylococcus aureus*, by Alexander Fleming in 1929, is well known and documented in general biological texts.[22] Fleming eventually grew the fungus in a liquid medium and, after separating the fluid from the cells, discovered that the cell free liquid was an inhibitor of many bacterial species. He called the active ingredient penicillin and this marked the discovery of antibiotics (substances produced by microorganisms that can kill or prevent the growth of other microorganisms). About ten years later Howard Florey and Ernst Chain successfully isolated and purified penicillin, and after being launched as a product in the early 1940s, it was soon saving lives from diseases such as pneumonia.

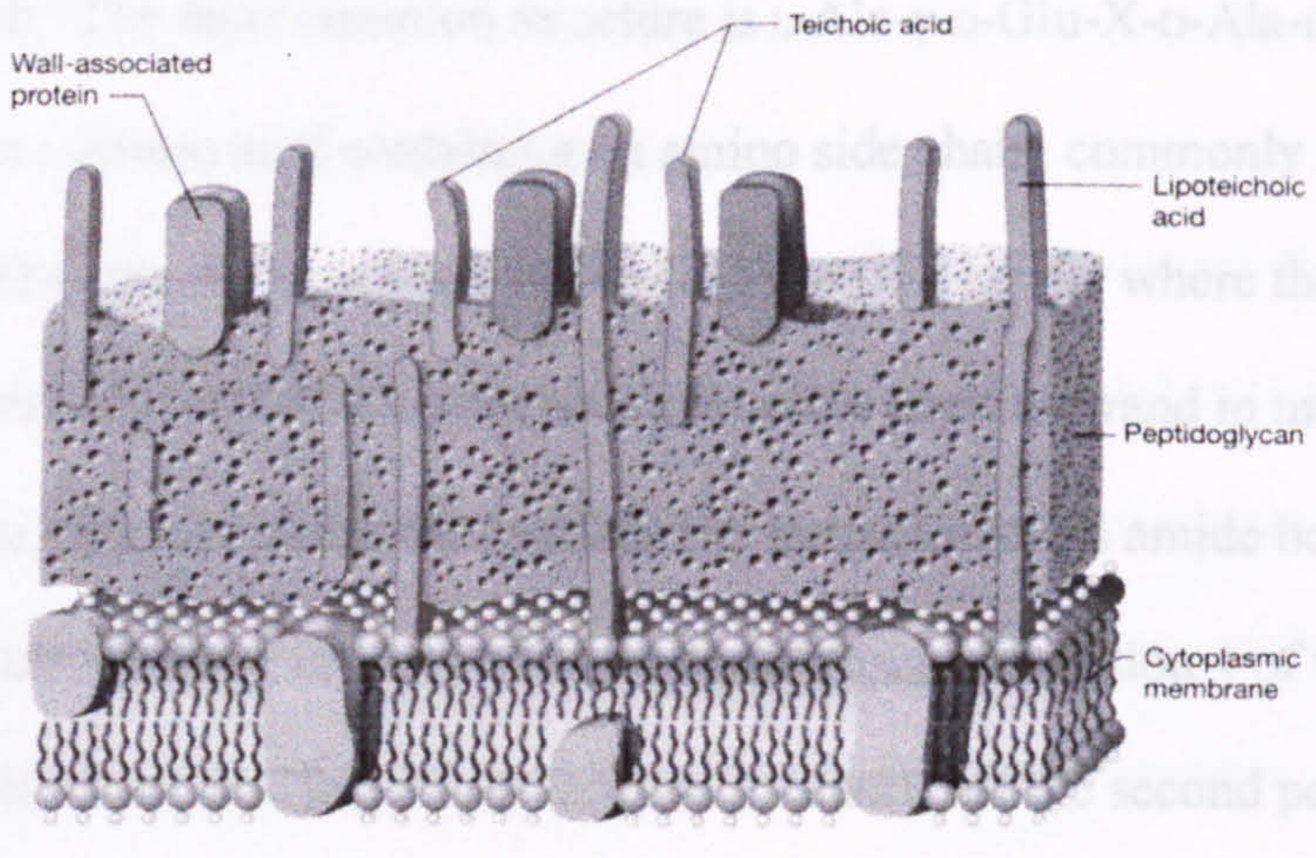
However, antibiotic resistance has developed. Resistance can take several forms where bacteria can alter the drug's target, prevent the drug from reaching the target by mechanisms such as efflux pumps or destroy or modify the drug so that it cannot bind to its target. This can occur when bacteria genetically mutate or acquire resistance genes from other bacteria, which can trade genetic material on pieces of DNA called plasmids. Misuse of antibiotics has generally contributed to the increase in resistance where for example antibiotics are prescribed for viral infections even though they do not kill viruses, or patients often fail to take all the antibiotic that has been prescribed. In these instances the most susceptible bacteria are killed but the more resistant strains survive. With less competition, the resistant bacteria have a more favourable environment in which to thrive, a

process known as selective pressure. A large amount of the antibiotics produced (possibly as much as 50%) are also fed to animals and fish, and sprayed on fruit trees. There is concern that antibiotic-resistant infections in humans may arise through foodborne illnesses caused by resistant bacteria or by the migration of resistance mechanisms between animal and human pathogens. It was estimated in the early 1990s that the unrecognised yearly cost of antibiotic resistance was $100 million and recently strains of glycopeptide-intermediate *S. aureus* (GISA) have shown reduced susceptibility towards vancomycin (the antibiotic of last resort). Fortunately the GISA strains have currently been susceptible to other antibiotics, although methicillin-resistant *S. aureus* bacteria are a concern due to their resistance to all antibiotics other than vancomycin. Encouragingly, a move to save essential antibiotics for human use is under way, for Rep. Sherrod Brown introduced a bill in the United States House of Representatives in November 1999 that would prohibit the use of essential antibiotics in livestock "unless there is a reasonable certainty of no harm to human health."[23]

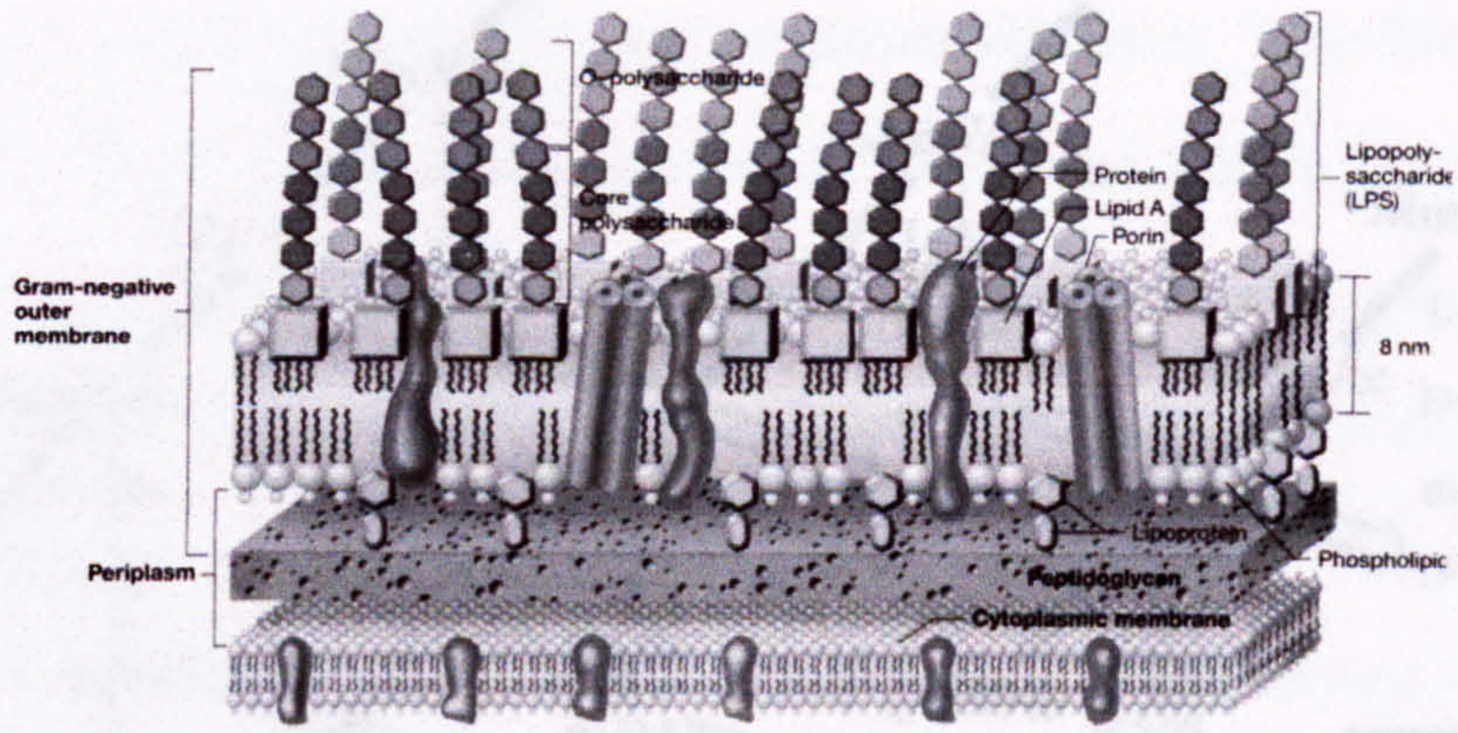
1.2.2 The Bacterial Peptidoglycan Layer

A detailed text by Bugg[24] provides an excellent review of the bacterial peptidoglycan, its biosynthesis and its inhibition. Bacteria generally fall into two main classes depending on whether their cell surfaces can be stained with a reagent discovered by Gram in 1884, namely Gram-positive and Gram-negative. The cell wall of almost all bacterial cells contain a peptidoglycan layer whose primary function is to strengthen the cell wall, preventing lysis through turgor

pressure which arises from a higher osmotic pressure inside the cell than outside. Gram-positive bacterial cell walls contain a thick outer layer of peptidoglycan (approximately 20-80 nm) which reacts strongly with Gram's reagent. Gram-negative bacterial cell walls are more complicated and possess an outer membrane which surrounds a thin peptidoglycan layer (approximately 2-3 nm) which limits the staining (Figure 1.9).



Gram-positive bacterial cell wall.



Gram-negative bacterial cell wall.

Figure 1.9. Gram-positive cell walls have a thick outer layer of peptidoglycan. Gram-negative cell walls have a thinner peptidoglycan layer encompassed with an outer membrane.

The structure of the peptidoglycan consists of three main components: disaccharide strands, pentapeptide chains and peptide cross-links (Figure 1.10). The glycan strands are generally made up of a β-1,4-linked chain of alternating N-acetylglucosamine and N-acetylmuramic acid residues, to which pentapeptide side chains are attached to the lactyl ether appendage of N-acetylmuramic acid. The pentapeptide chain contains D-amino acids that are unique to bacterial peptidoglycan. The most common structure is L-Ala-γ-D-Glu-X-D-Ala-D-Ala, where X is an L-amino acid containing an amino side chain, commonly L-lysine or *meso*-diaminopimelic acid (*meso*-DAP (*m*-DAP)). The site where the cross linkage occurs is through this amino acid to another peptide strand in mature peptidoglycan. Transpeptidation results in the formation of an amide bond between the amino group of X with the carbonyl group of position 4 of the second peptide chain, and loss of the terminal D-alanine of the second peptide chain.

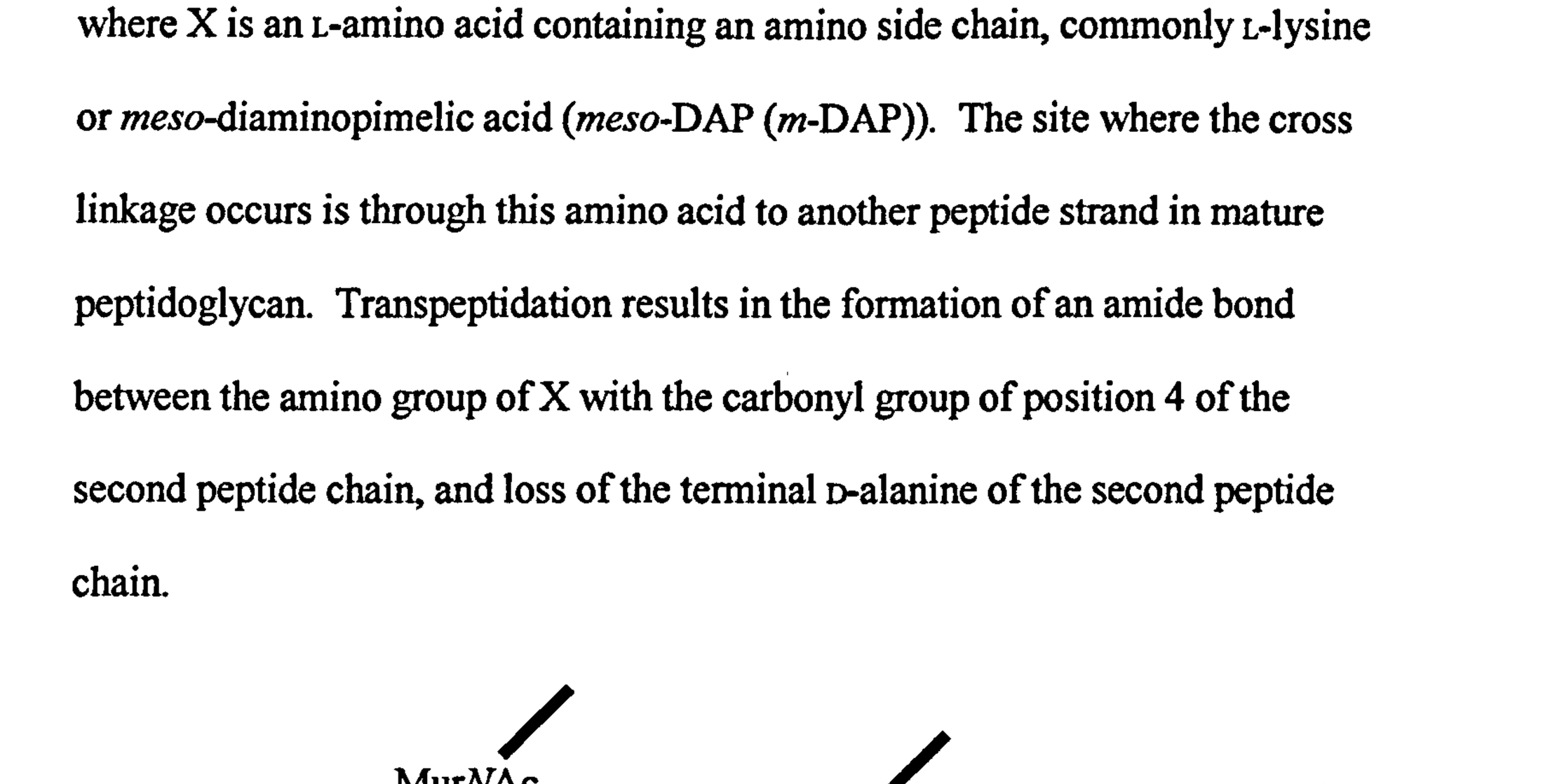


Figure 1.10. Generalised structure of mature peptidoglycan.

This eventually gives rise to the strong peptidoglycan structure, however it should be noted that the pentapeptide chain has some unique features, one of which is the presence of the D-amino acids which provide an avenue for selective inhibition of peptidoglycan biosynthesis.

1.2.3 Inhibition

During growth and division, the cell wall changes shape and size which requires the regulation of the peptidoglycan layer through a process which continuously breaks it down and rebuilds it. Certain enzymes, called autolysins, restructure or reshape the cell wall by breaking bonds in the peptidoglycan layer whilst others are responsible for the peptidoglycan biosynthesis. The latter enzymes are referred to as penicillin-binding proteins (PBPs) and it is to these proteins that penicillin covalently and irreversibly binds. PBPs are responsible for the biosynthetic cross-linking between the pentapeptide chains. Therefore, in this instance, the β-lactam antibiotic penicillin kills bacterial cells by inhibiting the enzymes which strengthen the peptidoglycan layer, whilst not effecting the enzymes which weaken it. The peptidoglycan layer eventually becomes weaker through a lack of pentapeptide cross linkage and the cell suffers lysis.

Vancomycin also kills bacterial cells by restricting the peptidoglycan's ability to form the cross-linkages between its pentapeptides. However, instead of inhibiting enzymes, vancomycin binds very strongly to the D-Ala-D-Ala portion of the pentapeptide and physically blocks the possibility of cross linkage.

1.2.4 Vancomycin Structure

Vancomycin was first isolated in 1956[25] from *Streptomyces orientalis* and its structure solved 25 years later.[26-29] Its defining feature is a linear heptapeptide backbone where the side chains of five amino acids are linked together to hold the peptide in a rigid conformation giving a cup-shaped architecture. The cavity created by this has a high binding affinity towards D-Ala-D-Ala, which is stabilised by a series of five hydrogen bonds (Figure 1.11).

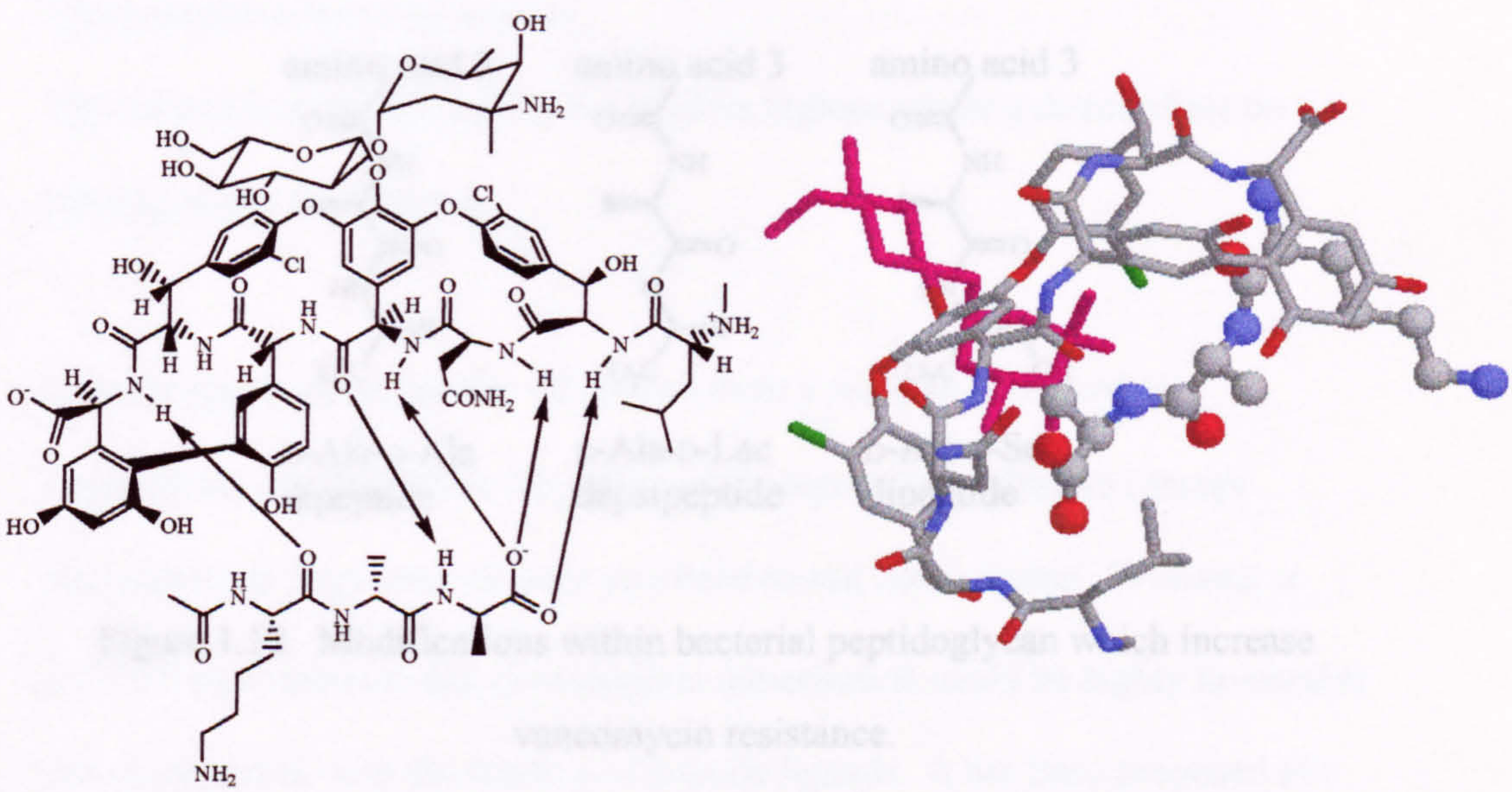


Figure 1.11. Left; indication of the hydrogen bonding between *N*-acyl-L-Lys-D-Ala-D-Ala and vancomycin. Right; a theoretical model of this complex.[2]

However, the cavity is also highly guest-specific. For example vancomycin's binding affinity to, di-*N*-acetyl-L-Lys-D-Ala-D-Ser is a factor of 20 weaker than that of di-*N*-acetyl-L-Lys-D-Ala-D-Ala[30] and the binding affinity for *N*-acyl-D-

Ala-D-Lac compared to *N*-acyl-D-Ala-D-Ala is a factor of 1000 less.[31] These particular models, along with others, were devised to investigate the binding affinities towards pentapeptide variations observed within the peptidoglycan layer of resistant bacteria. Strains of bacteria whose pentapeptide have evolved from L-Ala-D-Glu-L-Lys-D-Ala-D-Ala to L-Ala-D-Glu-L-Lys-D-Ala-D-Lac are particularly resistant to vancomycin[32] (Figure 1.12).

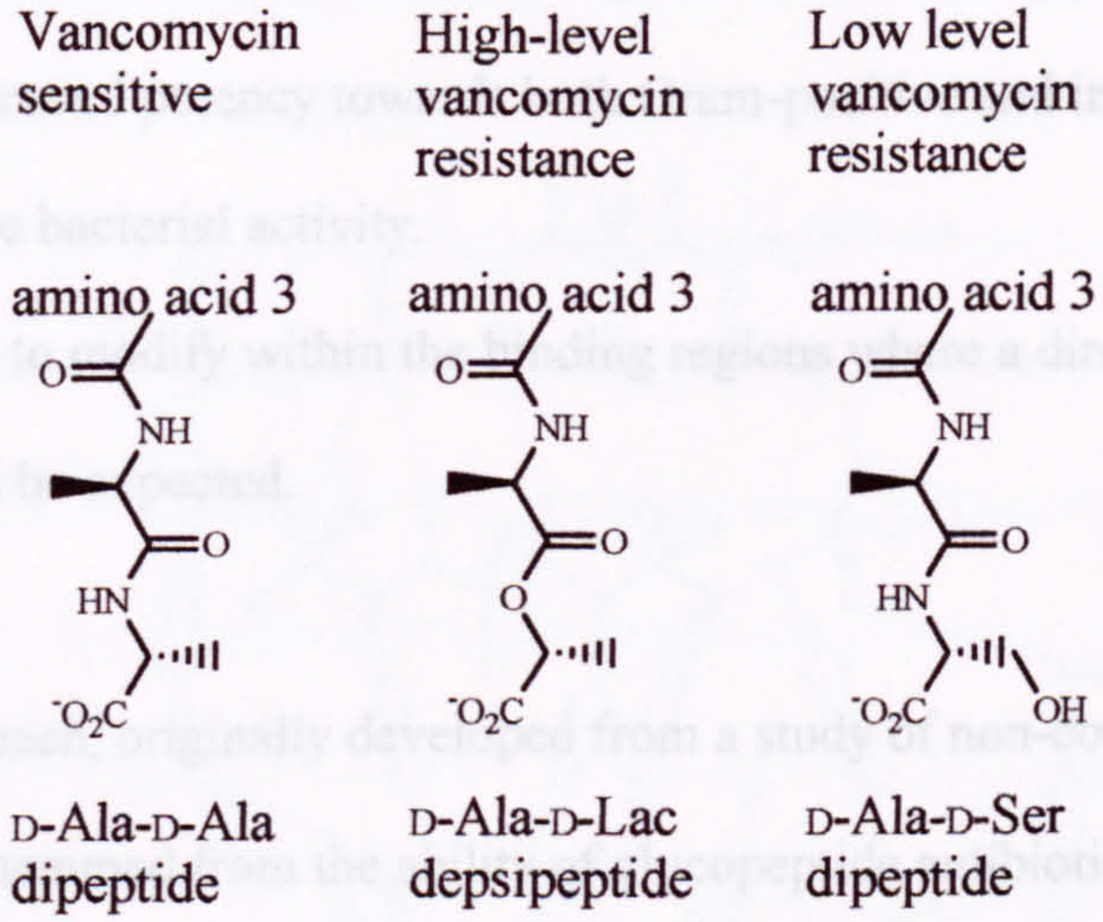


Figure 1.12. Modifications within bacterial peptidoglycan which increase vancomycin resistance.

1.2.5 Modifications to Increase Vancomycin Activity

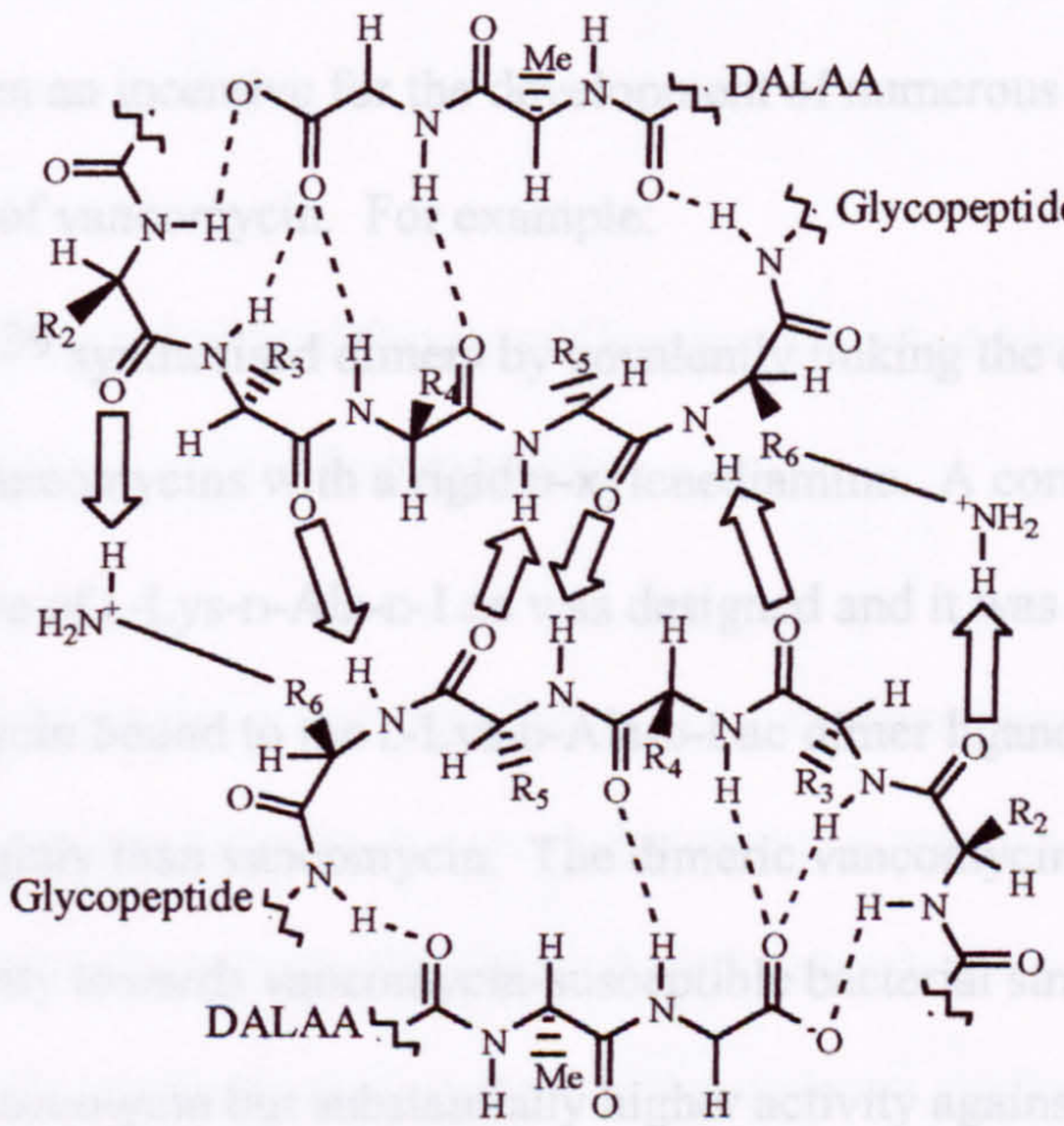
Two approaches have been taken to enhance activity of glycopeptide antibiotics such as vancomycin or teicoplanin through synthetic modifications and these have been reviewed.[33] The first is the modification of sites that do not directly

impinge on the binding cavity. These include removal or substitution of sugars, the modification of accessible functional groups such as the carboxy group of amino acid 7, certain N-terminal modifications and derivation of hydroxy groups. In this instance two examples of successful approaches to improved antimicrobial activity without direct alteration of the binding site, have been reported. These are: a) *N*-alkyl modifications of the amino sugars of a vancomycin-related glycopeptide and b) the modification of the terminal carboxyl by amide derivatisation of teicoplanin and related glycopeptides, affording improved potency towards both Gram-positive and in some instances Gram-negative bacterial activity.

The second is to modify within the binding regions where a direct effect on binding might be expected.

Another approach, originally developed from a study of non-covalent interactions, stemmed from the ability of glycopeptide antibiotics (except teicoplanin) to form back-to-back and head-to-tail homodimers. Williams *et al.*[34-36] demonstrated that glycopeptide dimerisation could be highly favourable and co-operative with the binding of peptide ligands. It has been proposed this could be due to two reasons.[37] The first appears to be motional restriction of the glycopeptide. For the monomer, when the amide functional groups of the glycopeptide backbone are hydrogen bonded to water, they would probably have more motional freedom than when hydrogen bonded to each other as in the dimer. The ordering of the amide functional groups should promote ligand binding since the magnitude of hydrogen bond fluctuations at the ligand-glycopeptide interface would be reduced. Secondly, the ring 6 amino sugar,

where present, is proposed to form an ammonium ion-amide carbonyl hydrogen bond in the dimer, to residue 2 of the complementary subunit (Figure 1.13). This may polarise the NH proton of this residue 2-3 amide linkage so that it forms a stronger hydrogen bond to the ligand carboxylate ion in a complex. This implies that for the ligand bound dimer, the alkylammonium ion of the amino sugar on residue 6 of the antibiotic is able to form an indirect salt bridge to the carboxylate anion of the bacterial cell wall analogue, mediated through the polarisable amide bond which connects residues 2 and 3 of the antibiotic.



DALAA = di-*N*-actyl-L-Lys-D-Ala-D-Ala

Figure 1.13. Hydrogen bonding interactions shown within back-to-back ligand bound dimers.

Vancomycin itself only weakly self-associates in solution, suggesting that dimerisation may not play a significant role in its biological action. For this reason Griffin and Sundran synthesised covalently linked dimers of vancomycin

to examine how their molecular and antibacterial properties would be affected.[38] Indeed certain dimers were shown to display enhanced *in-vitro* potency against strains of enterococci which exhibited high-level resistance to vancomycin and other glycopeptides and these studies were also substantiated with tripeptide ligand binding studies. However, these dimers were covalently linked through flexible linkers that were too short to allow interactions between the vancomycin sub-units in the back-to-back, head-to-tail orientations. Rather the head-to-head orientation was favoured. Therefore it was envisaged that different types of divalent interactions with ligands in the bacterial wall were occurring. This evidence has been an incentive for the development of numerous synthetic covalent dimers of vancomycin. For example:

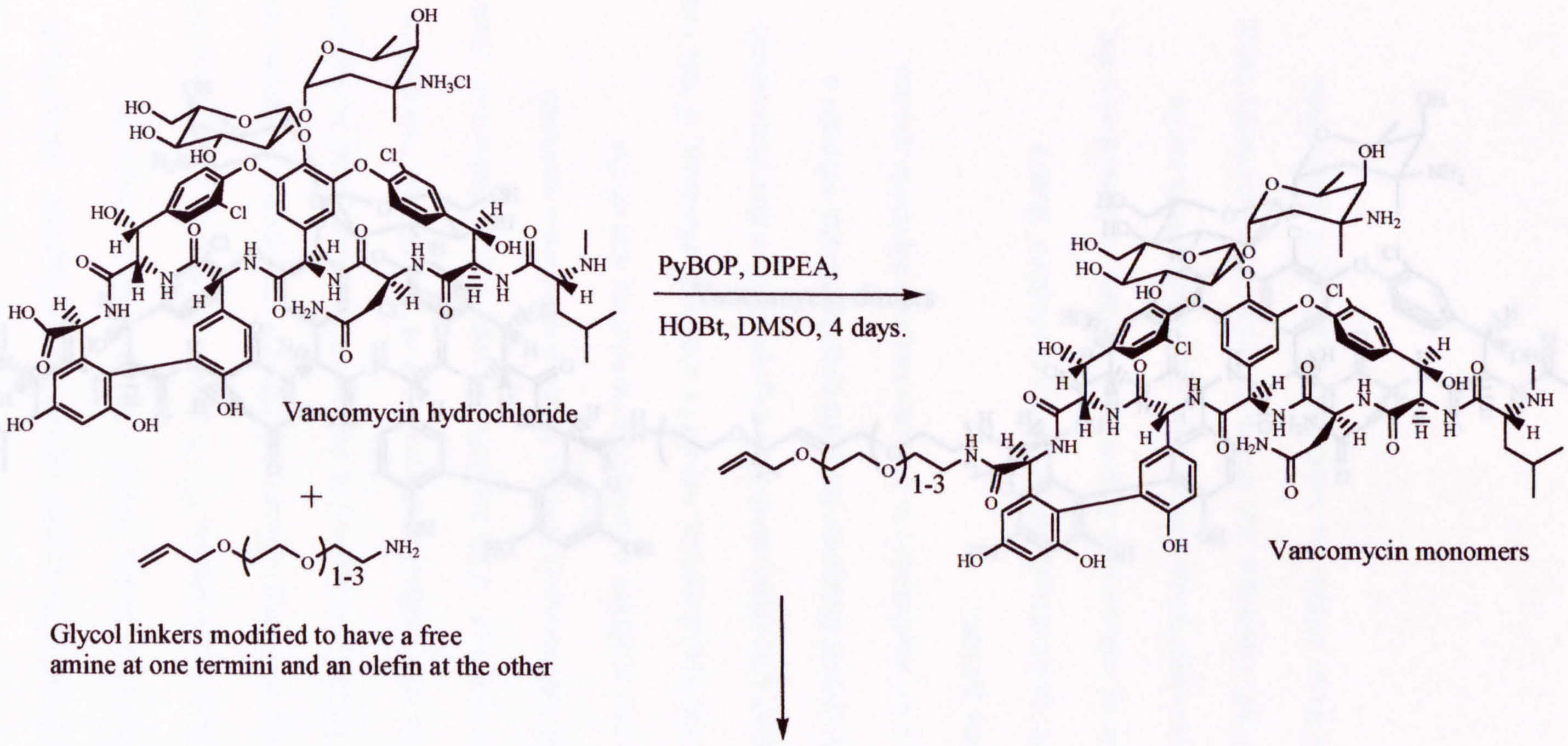
Whitesides *et al.*[39] synthesised dimers by covalently linking the carbonyl termini of two vancomycins with a rigid *p*-xylenediamine. A corresponding dimeric derivative of L-Lys-D-Ala-D-Lac was designed and it was shown that dimeric vancomycin bound to the L-Lys-D-Ala-D-Lac dimer ligand approximately 40 times more tightly than vancomycin. The dimeric vancomycin also showed comparable activity towards vancomycin-susceptible bacterial strains normally achieved with vancomycin but substantially higher activity against vancomycin-resistant strains. Whitesides *et al* [40] have recently shown that a high affinity exists between a vancomycin trimer and an analogous trivalent derivative of D-Ala-D-Ala.

A polymer of vancomycin was synthesised *via* a ring opening metathesis polymerisation and this was shown to exhibit an enhanced of antibacterial activity against vancomycin resistant *enterocci.*[41]

Williams and Heck[42] showed the presence of heterodimers in one-to-one mixtures of antibiotics by detection with elecrospray ionisation mass spectrometry (ESI-MS) and these were confirmed to exist in aqueous solution using NMR spectroscopy. In the instance of a mixture of eremomycin and chloroeremomycin, the ion signal due to the heterodimer was the most intense observed. However, investigations undertaken to determine the minimum inhibitory concentrations (MICs) of one-to-one mixtures of antibiotics indicated that heterodimer formation did not give a significant improvement in biological activity relative to the pure antibiotics.

1.2.6 Screening a Combinatorial Range of Covalently Linked Vancomycin Dimers, For Activity *via* Mass Spectrometry

In view of the evidence that it was possible to increase vancomycin activity by forming covalently linked dimers, we wanted to design a model to investigate the effect of combinatorially varying the linkers length towards their activity (Scheme 1.11). We envisaged that vancomycin monomers synthetically modified at the carboxy group of amino acid 7 with glycol linkers of varying lengths attached through an amide bond, bearing a terminal double bond, could be combinatorially coupled *via* Grubbs metathesis (Figure 1.14).



Scheme 1.11. Vancomycin monomers modified with variable glycol linkers expressing a terminal double bond.

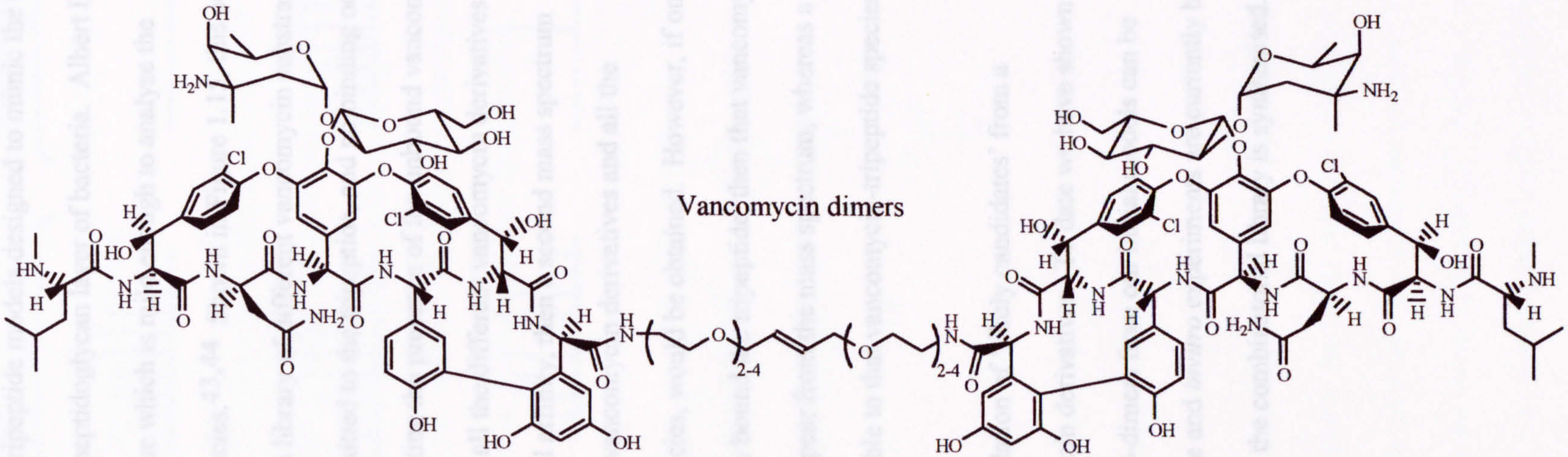


Figure 14. A combinatorial range of vancomycin dimers created *via* Grubb's metathesis.

As described above, previous studies with vancomycin and vancomycin dimers have been shown to bind di and tripeptide models designed to mimic the terminal of the pentapeptide found in the peptidoglycan layer of bacteria. Albert Heck has designed an ESI-MS technique which is mild enough to analyse the vancomycin-tripeptide bound species.[43,44] Shown in Figure 1.15, this could be a useful technique for analysing a library of different vancomycin substrates and dimers. When the library is submitted to the tripeptide, and no binding occurred then a mass spectrum only indicating the presence of the unbound vancomycin derivative would be observed. If all the different vancomycin derivatives bound to the tripeptide and with an equal affinity, then a second mass spectrum indicating the presence of all the vancomycin derivatives and all the vancomycin-tripeptide bound species, would be obtained. However, if only one vancomycin derivative selectively bound the tripeptide, then that vancomycin derivative would be seen to disappear from the mass spectrum, whereas a corresponding new peak attributable to that vancomycin-tripeptide species, would clearly become present.

This would allow the quick elucidation of 'likely candidates' from a combinatorial library of vancomycin derivatives. To date we have shown the vancomycin monomers and homo-dimers from our initial models can be analysed by the ESI-MS technique and *invitro* experiments are currently being undertaken to verify these, before the combinatorial library is synthesised.

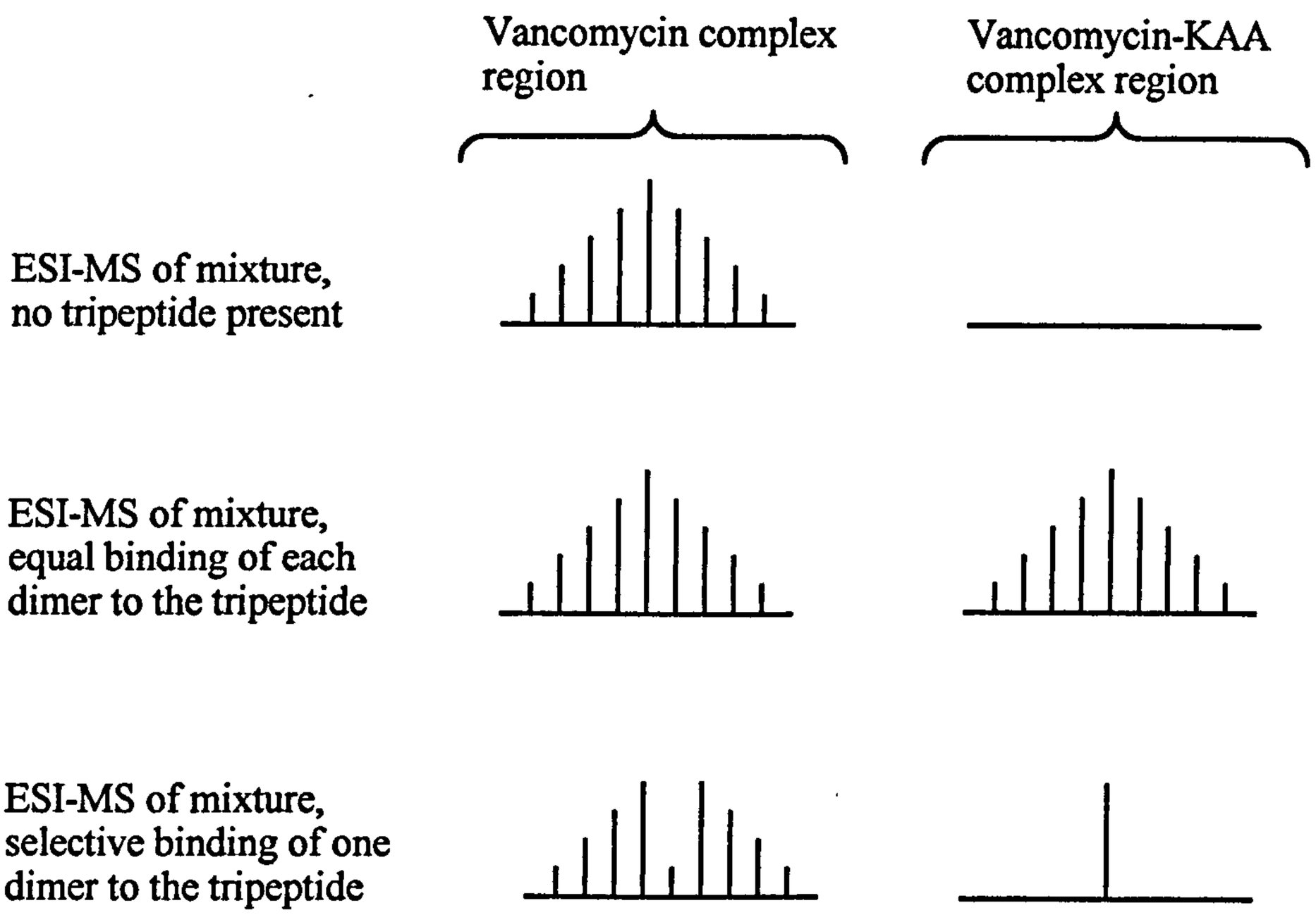


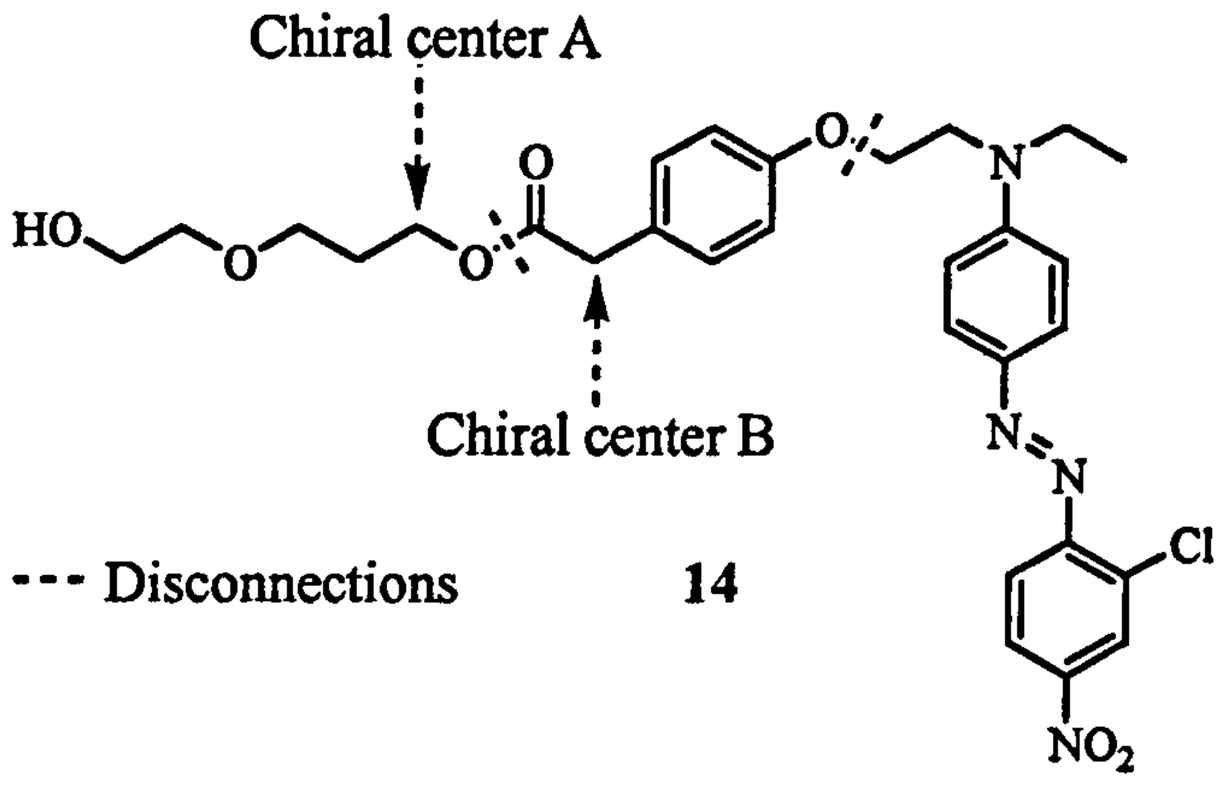
Figure 1.15. A vancomycin derivative's ability to bind the tripeptide model can be investigated by analysing its increase in mass.

2 Results and Discussion

2.1 Synthetic Esterase

2.1.1 Initial Development of The Dye-Linker System

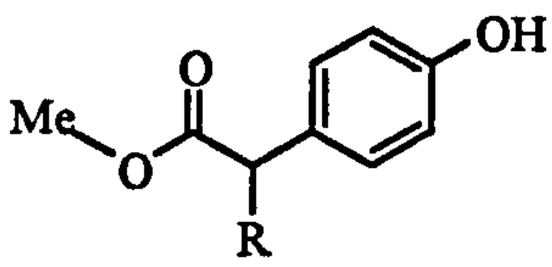
A major component of the model we wished to develop in order to probe whether small linear peptides could be used to hydrolyse ester bonds, was the "dye-linker" system. The refined dye-linker system is shown in Figure 1.7, (previous chapter). Initially, however, a simpler dye-linker **14** was chosen to allow the synthetic methodology to be firmly established (Scheme 2.1).



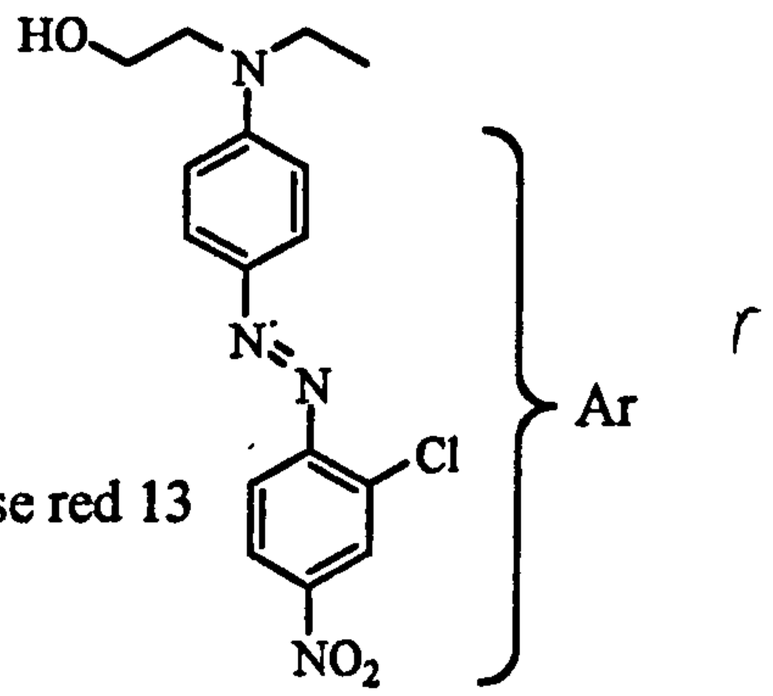
Scheme 2.1. The original dye-linker **14** was designed to allow the addition of chiral centers at positions A and B.

The original dye-linker **14** contained a short ethoxy-propoxy chain **15** coupled through 4-hydroxyl phenyl acetic acid to the highly coloured red azo dye, disperse red (Scheme 2.2). The retrosynthetic disconnections employed for the synthesis of the original dye-linker are shown in Schemes 2.1 and 2.2. Two positions of interest were A and B, where chiral centers could be introduced at a

later stage to allow the investigation of potential enantioselective control delivered by active synthetic esterases. It was envisaged that a chiral center at position A could be introduced whilst synthesising the ethoxy-propoxy chain **15** *via* asymmetric hydroboration and oxidative work up of various analogues (R^1 = alkyl, aryl, halide). A chiral center at position B could be introduced *via* asymmetric analogues of methyl 4-hydroxyphenyl acetate (R = alkyl, aryl, halide).

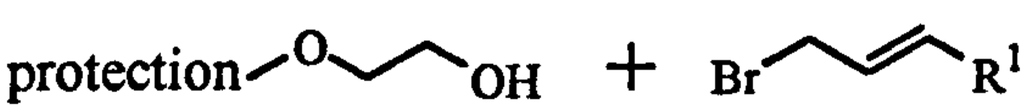


methyl 4-hydroxyphenyl acetate, R = H



disperse red 13

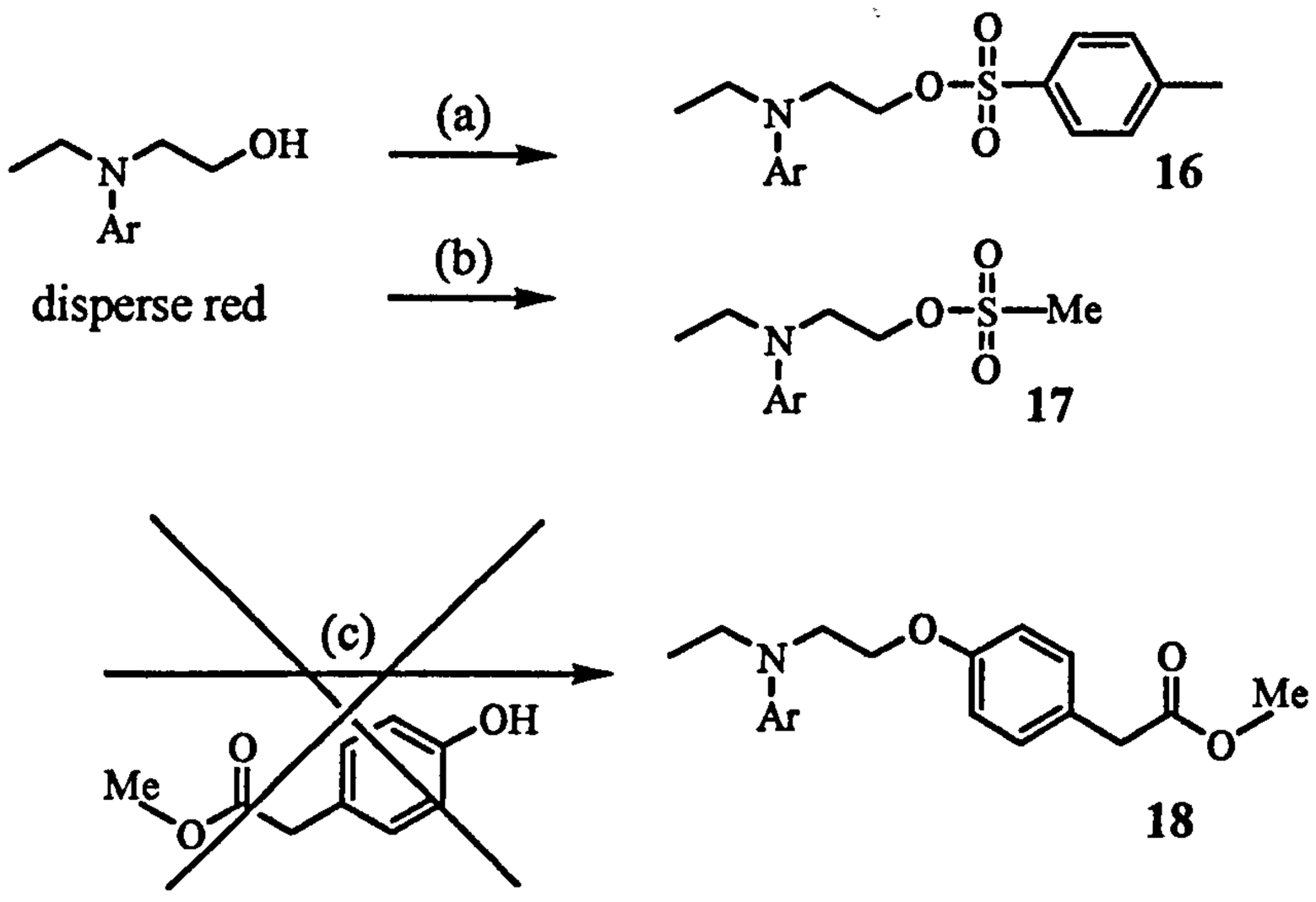
R^1 = H, **15**



Scheme 2.2. Retrosynthetic starting materials of the original dye-linker **14**.

The first stage of the synthesis of dye-linker **14** involved coupling disperse red to methyl 4-hydroxyphenyl acetate (Scheme 2.3). The hydroxy group of disperse red was converted into a good leaving group by conversion to firstly its tosylate[45] **16** and secondly, to its mesylate[46] **17**. It was hoped direct nucleophilic displacement of either of these groups by the hydroxyphenyl of

methyl 4-hydroxyphenyl acetate would form the protected methyl ester **18**. Unfortunately, the tosylate and mesylate derivatives of disperse red proved to be unstable to the anhydrous basic conditions employed to effect nucleophilic displacement, and the product was never formed.

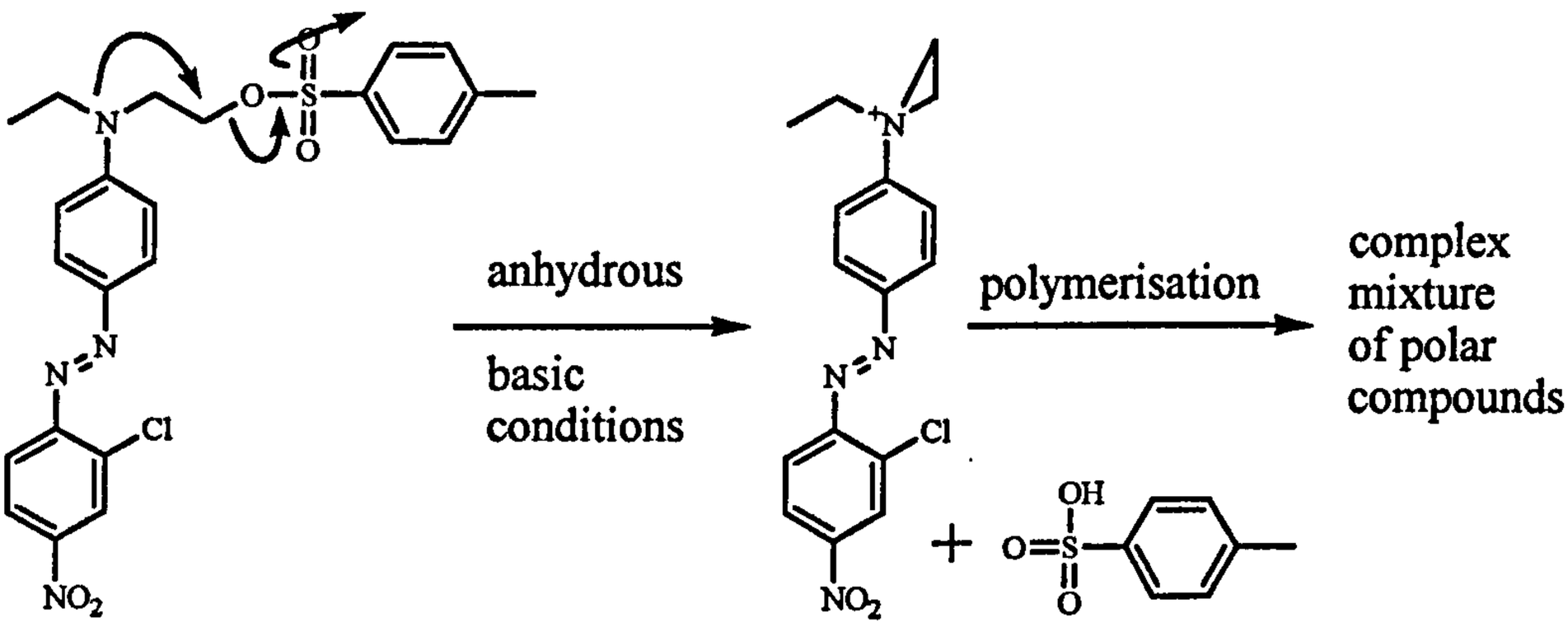


(a) Disperse red (1 eq), *p*-toluene sulfonyl chloride (2.2 eq), triethylamine (3 eq), dichloromethane, room temperature, 8 hours, 74%. (b) Disperse red (1 eq), methane sulphonyl chloride (2.2 eq), triethylamine (3 eq), dichloromethane, room temperature, 6 hours, 80%. (c) **16** or **17** (1 eq), 4-hydroxyphenylacetic acid (1.1 eq), base (1.2 eq), tetrahydrofuran, room temperature.

Scheme 2.3. Nucleophilic displacement of either disperse red tosylate **16** or mesylate **17** resulted in a complex mixture of polar compounds, under basic conditions.

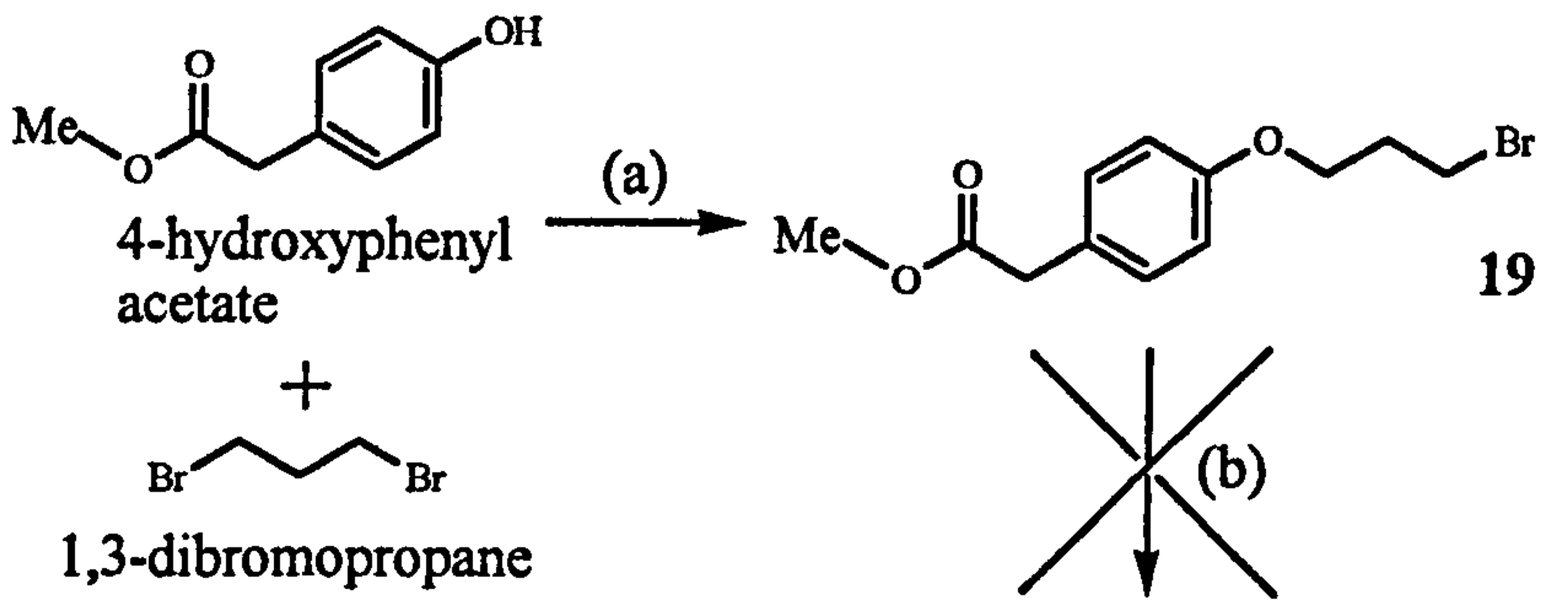
Several bases were investigated and these included potassium carbonate, sodium hydride, potassium hydride and *tert*-butyl lithium. When *tert*-butyl lithium was employed, TLC indicated the disperse red derivatives were quickly converted to a complex mixture of polar compounds. The other bases afforded the same

complex mixture of polar compounds, but took several hours. The ^{1}H nuclear magnetic resonance (NMR) of the crude reaction mixture was complicated and the presence of product was not indicated. The difficulty of achieving the nucleophilic displacement was surprising and could have been accountable to the leaving group being *beta* to the *tert*-amine in disperse red (Scheme 2.4). Initial formation of an aziridonium ring could cause polymerisation of the disperse red giving rise to a complicated mixture of polar products.



Scheme 2.4. Formation of an aziridonium ion *via* β-elimination could have given polymerisation.

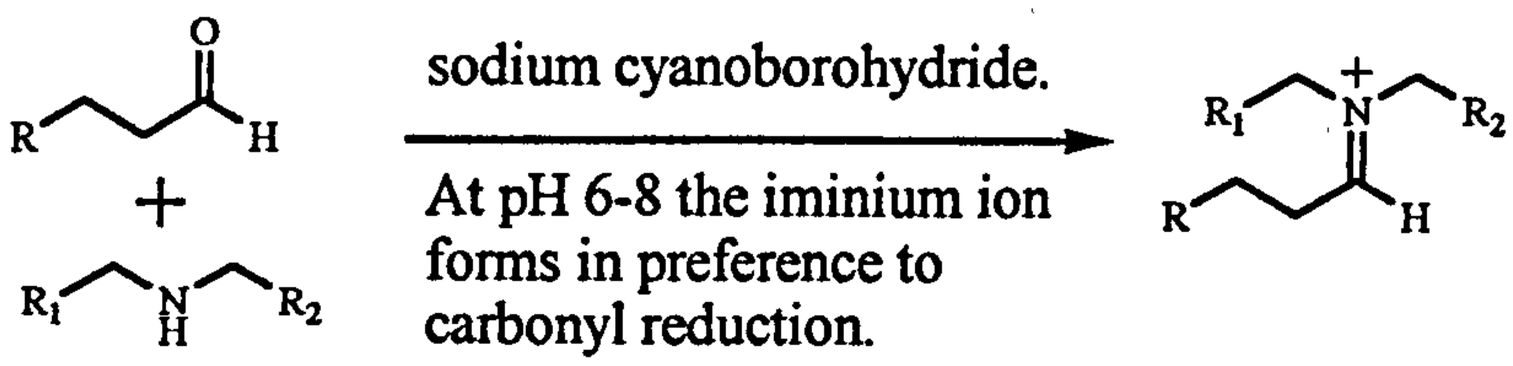
Another attempt to form a similar analogue to **18** was undertaken. Methyl 4-hydroxyphenyl acetate was converted to **19** upon nucleophilic displacement with 1,3-dibromopropane in reasonable yield (Scheme 2.5). Displacement of the bromide of **19**, by disperse red under anhydrous basic conditions, was attempted. Although a complex mixture of polar compounds was not observed, regrettably after 48 hours no conversion to **20** was observed.



(a) Methyl 4-hydroxyphenyl acetate (1 eq), 1,3-dibromopropane (2 eq), sodium hydride (1 eq), tetrahydrofuran 12 hours, 42%. (b) Disperse red (1 eq), **19** (1.2 eq), base (1.2 eq), tetrahydrofuran, room temperature, 12 hours.

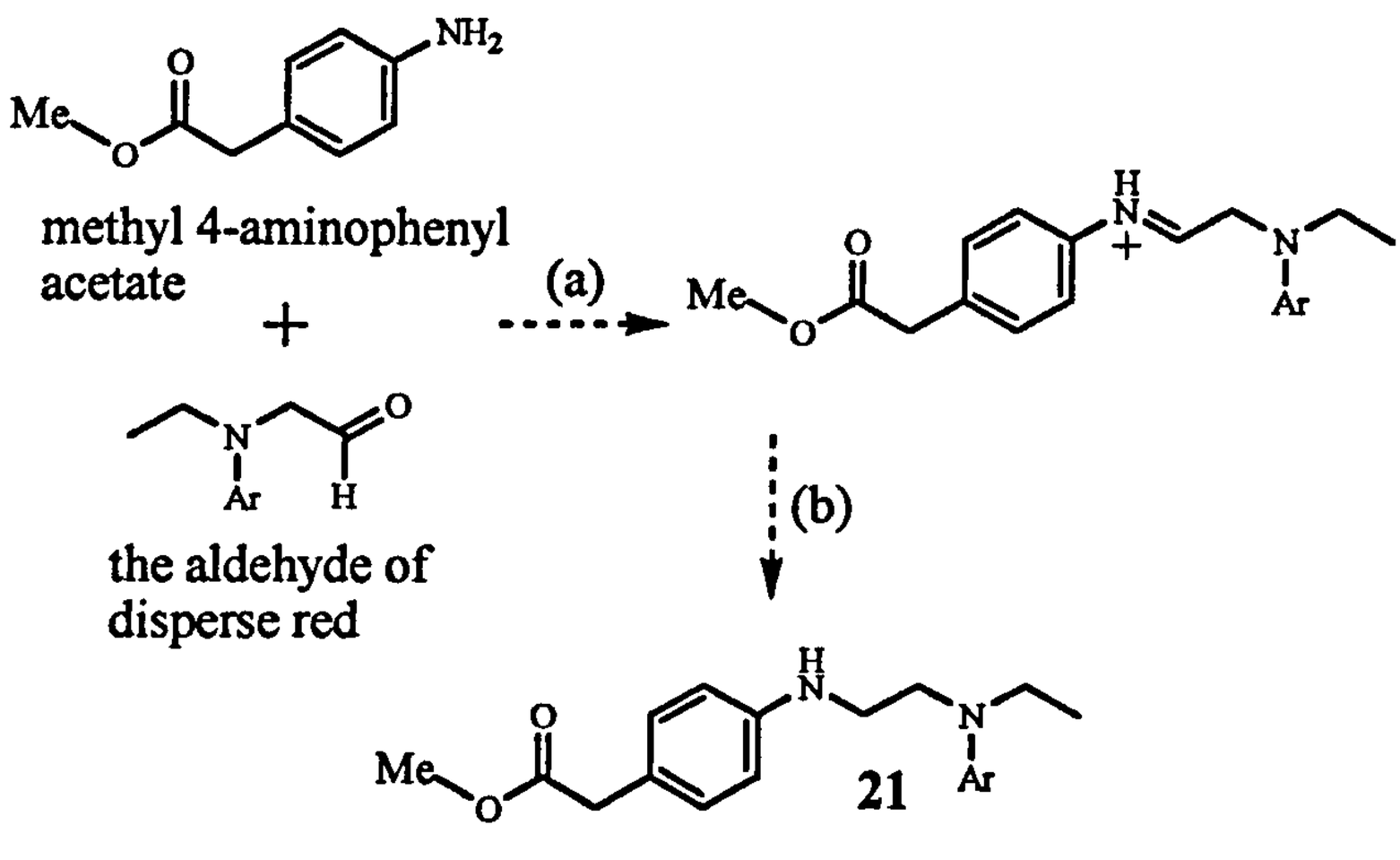
Scheme 2.5. Disperse red was found to be stable to the anhydrous basic conditions, however nucleophilic displacement of the bromide did not occur.

Having found that disperse red did not readily effect nucleophilic displacement of the bromide of **19**, and its derivatives **16** and **17** were unstable to the anhydrous basic conditions, a different approach utilising reductive amination with sodium cyanoborohydride was investigated. Sodium cyanoborohydride is a useful reagent for achieving reductive amination[47] because it readily reduces carbonyl compounds at pH 3-4 whereas it quickly reduces iminium ions at pH 6-8 (Scheme 2.6).



Scheme 2.6. Reductive amination *via* sodium cyanoborohydride

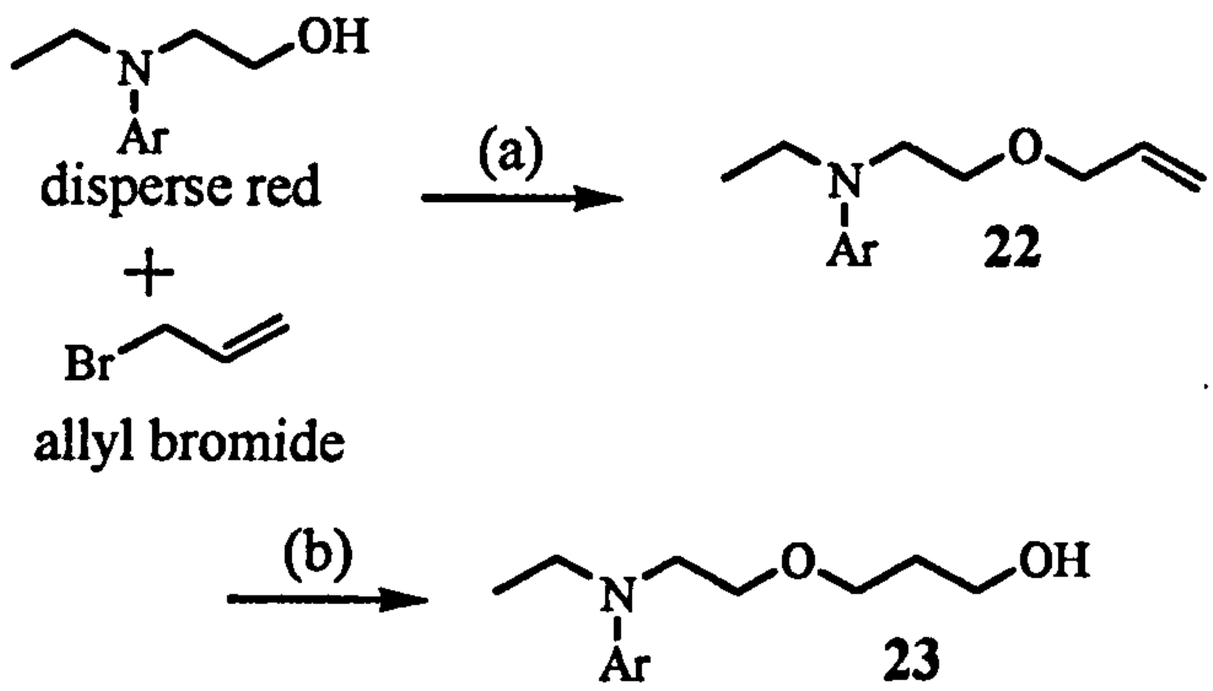
To effect the coupling of disperse red to methyl 4-aminophenyl acetate *via* reductive amination to give **21**, the procedure shown in Scheme 2.7 was proposed. However this required the alcohol function of disperse red to be oxidised to the aldehyde.



(a) Formation of the iminium ion in tetrahydrofuran. (b) Reduction by sodium cyanoborohydride.

Scheme 2.7. Coupling *via* reductive amination.

However, the oxidation of disperse red was problematic and a second derivative, **23** bearing a propoxy chain, was investigated simultaneously (Scheme 2.8).



(a) Disperse red (1.0 eq), allyl bromide (1.2 eq), sodium hydride (1.0 eq), cesium carbonate (catalytic), tetrahydrofuran, room temperature, 4 hours, 97%. (b) **22** (1.0 eq), borane methylsulfide (3.0 eq), toluene, room temperature, 3 hours. Methanol (excess), hydrogen peroxide (2.0 eq), sodium hydroxide (2.5 eq), room temperature, 2 hours, 40%.

Scheme 2.8. The primary alcohol function of disperse red was extended through a propoxy chain.

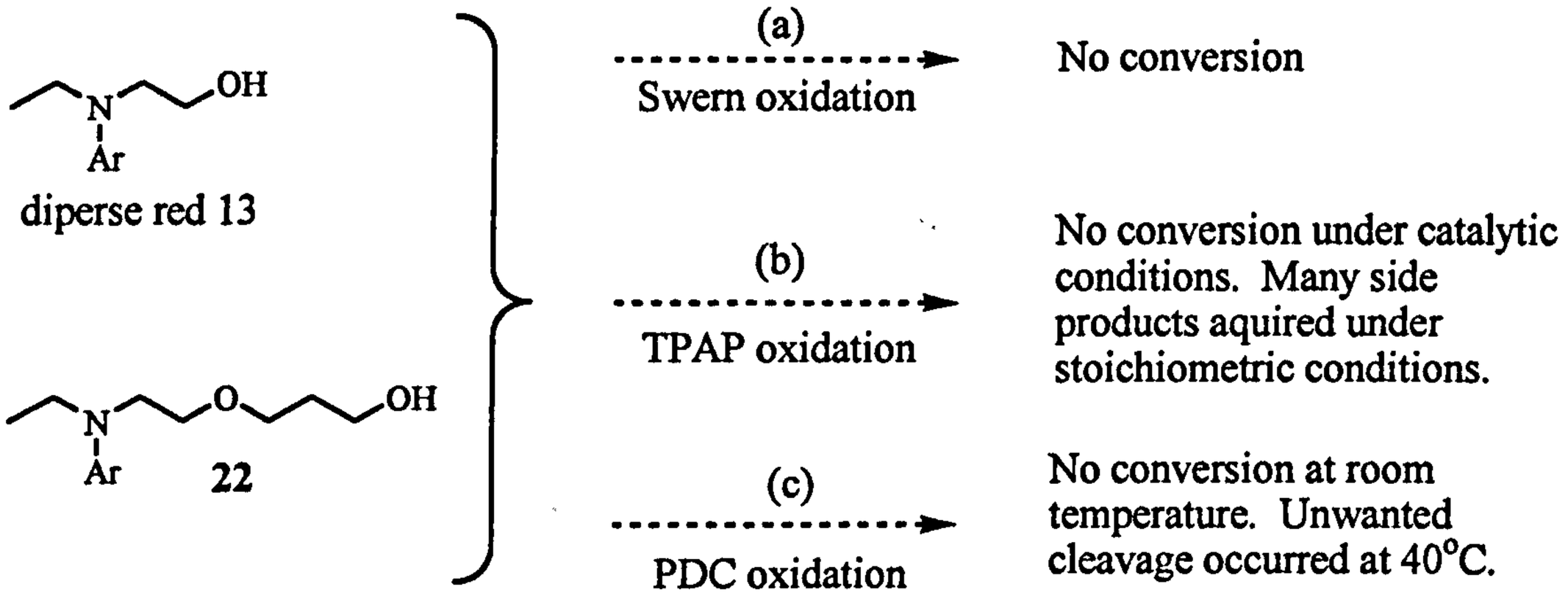
The alcohol function was extended by allylation of disperse red to give **22**, followed by hydroboration with borane dimethyl sulfide[48] and oxidative work up with hydrogen peroxide and sodium hydroxide to give **23**. Previously it had not been possible to displace the bromide of **19** by disperse red and it was also difficult to displace the bromide of allyl bromide. Favourable conditions involved the use of the dipolar aprotic solvent dimethylformamide to encourage nucleophilic attack, with sodium hydride as base giving a moderate yield of 67% over a period of 18 hours.

However, the protocol was later improved by the addition of cesium carbonate, giving full conversion within 1 hour where the yield was improved to 97%. Cesium carbonate is known to aid the esterification of protected amino acids by alkyl halides in dimethylformamide[49] and the di-cesium salts of catechol and resorcinol are also reported to be superior to other alkali metal salts in the reaction with dibromoethers in dimethylformamide, to give crown ethers.[50]

Disperse red and **23** were subjected to Swern[51], tetra-n-propylammonium perruthenate (TPAP)[52,53] and pyridinium dichromate (PDC)[54] oxidative conditions (Scheme 2.9). Regrettably, no conversion to of either disperse red or **23** was observed during the Swern oxidation conditions. Crude ^{1}H NMR revealed only the presence of starting material.

TPAP was initially used in catalytic amounts with 4-methylmorpholine N-oxide (NMO) which was the standard oxidising agent required to regenerate TPAP during its catalytic cycle. In both instances only partial immediate conversion was observed giving no further conversion after several hours. TPAP was also investigated on a smaller scale to see if a reasonable conversion could be achieved using a stoichiometric amount of reagent. Full conversion to a complex mixture of polar compounds was observed within 1 hour. However, TLC (ethyl acetate:hexane, 1:4) did not reveal the presence of the product and the ^{1}H NMR of the crude reaction mixture was complicated.

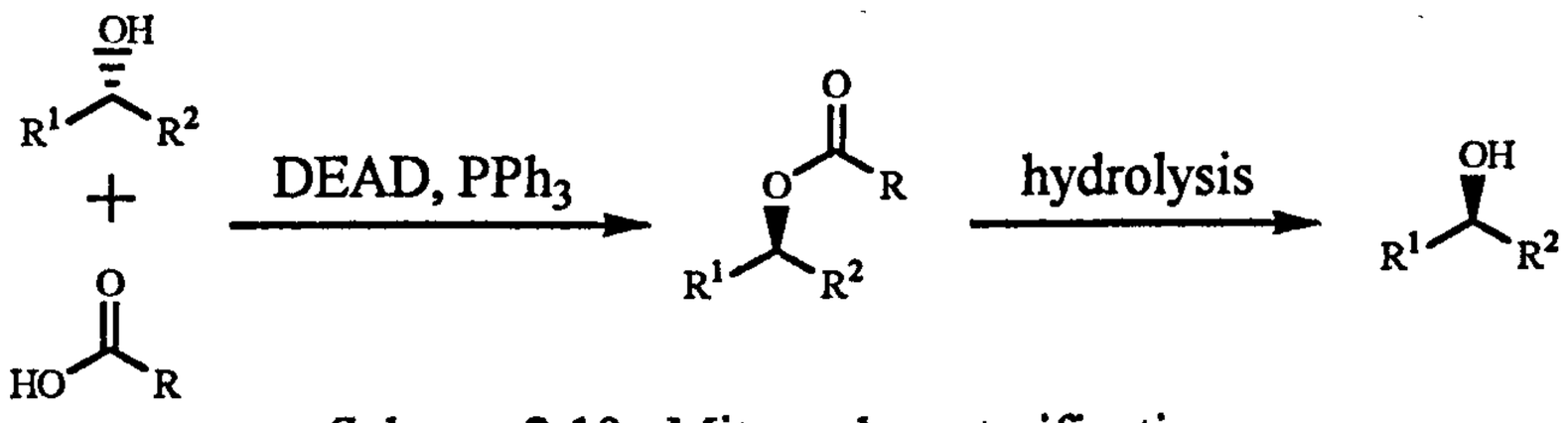
The PDC oxidative reactions were undertaken using dichloromethane as solvent. It is known from the literature precedent[54] that PDC converts primary alcohols to their corresponding aldehydes in aprotic solvents. However, if further oxidation to the carboxylic were desired, then a more-polar solvent such as dimethylformamide was advised. Again no conversion for either starting material was observed for several hours. However, when the oxidation of disperse red was heated to 40°C, full conversion to a new product within 20 minutes occurred and the solution changed its colour from dark red to light orange. Unfortunately the ^{1}H NMR of the crude reaction mixture revealed the oxidative conditions had cleaved the ethyl group from the *tert*-nitrogen of the dye.



(a) Alcoholic substrate (1.0 eq), oxalyl chloride (1.1 eq), dimethyl sulfoxide (1.1 eq), triethylamine (1.5 eq), tetrahydrofuran, -78°C to room temperature, 10 hours. (b) Alcoholic substrate (1.0 eq), TPAP, NMO (1.1 eq), molecular sieves, tetrahydrofuran, room temperature, 10 hours. (c) Alcoholic substrate (1.0 eq), PDC (1.1 eq), dichloromethane, room temperature.

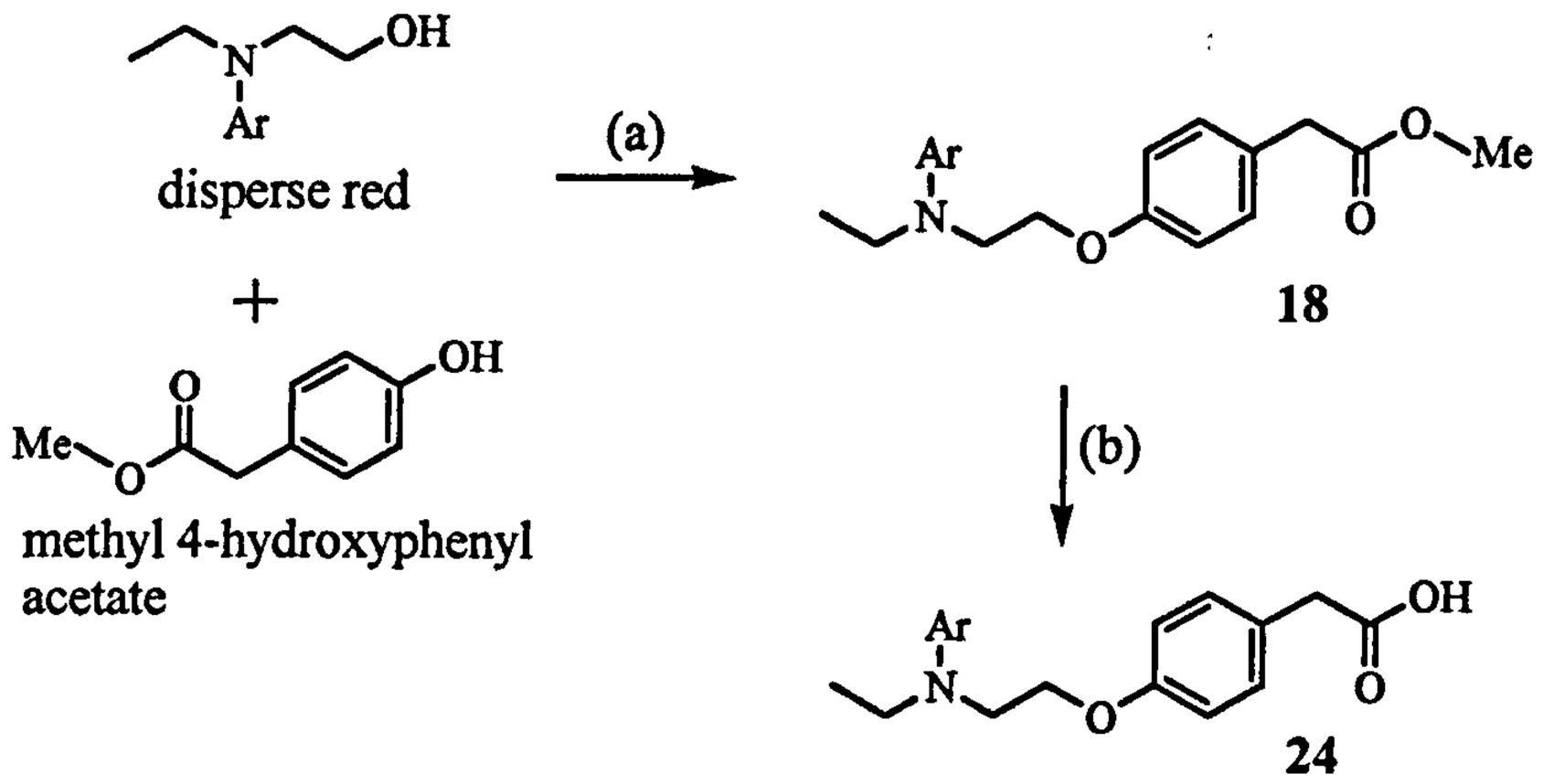
Scheme 2.9. Attempted oxidation of disperse red and **22** *via* Swern, TPAP and PDC oxidation.

Disperse red was proving to be difficult to manipulate so a Mitsunobu alkylation was considered. The Mitsunobu reaction[55-57] follows an S_N2 mechanism and is quite often applied in natural product synthesis to convert a chiral secondary alcohol into an ester *via* Mitsunobu esterification, with concomitant inversion of configuration at the secondary carbon center. Subsequent hydrolysis of the ester yields the original alcohol but as the opposite enantiomer (Scheme 2.10).



Scheme 2.10. Mitsunobu esterification.

The Mitsunobu alkylation between disperse red and methyl 4-hydroxyphenyl acetate, is shown in Scheme 2.11. Initially the formation of the disperse red dimer predominated the overall reaction, therefore the reagents order of addition was investigated. It was known that it was possible to control the Mitsunobu reaction to a greater degree by allowing the phosphonium ion to form before the final addition of one of the starting materials. In this instance, it was found that if the reaction was performed in toluene at 80°C and disperse red was added last after stirring the other reagents at 80°C for 10 minutes, full conversion to the product in reasonable yield could be achieved within 30 minutes.



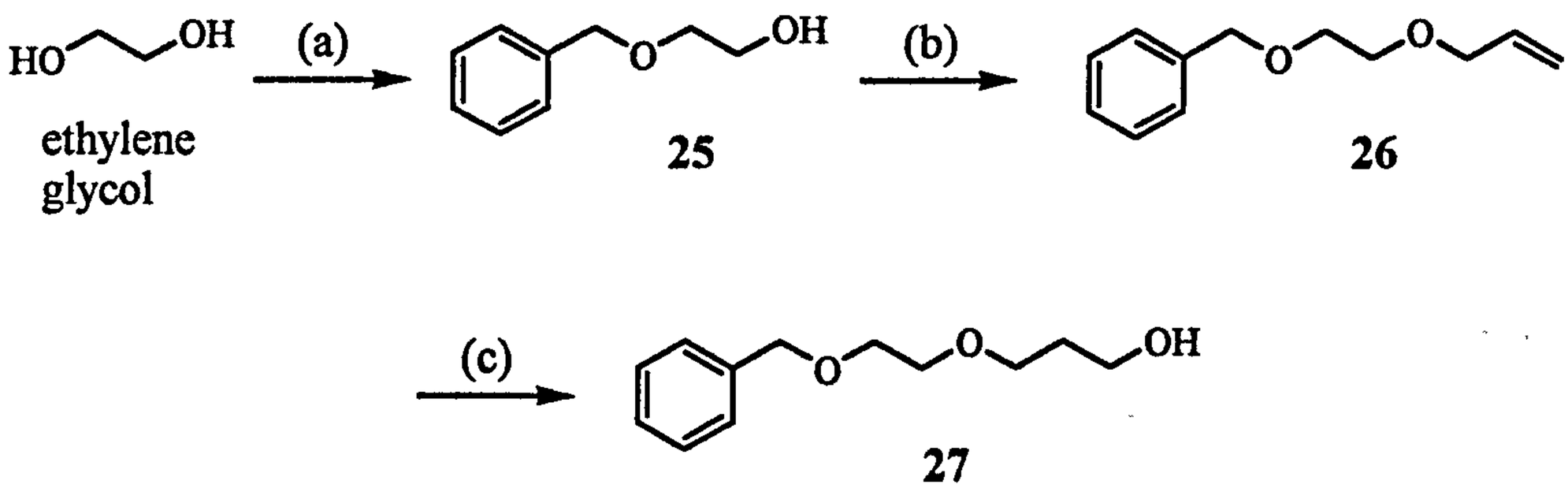
(a) Disperse red (1.0 eq), methyl 4-hydroxyphenyl acetate (2.0 eq), diethyl azodicarboxylate (DEAD) (2.0 eq), triphenylphosphine (PPh_3) (2.0 eq), toluene, 80°C, 58%. (b) **18** (1.0 eq), lithium hydroxide (2.0 eq), water:tetrahydrofuran, 1:15, 45°C, 30 minutes, 79%.

Scheme 2.11. Mitsunobu alkylation between disperse red and methyl 4-hydroxyphenyl acetate.

Once **18** had been formed, the methyl ester was saponified using lithium hydroxide in wet tetrahydrofuran[58] to give **24** in 79% yield.

The next stage towards the synthesis of the original dye-linker **14** was formation of the benzyl protected linker **27** (Scheme 2.12). Compound **27** was synthesised from ethylene glycol by mono-benzylation, allylation and hydroboration with an oxidative workup. To avoid double protection, ethylene glycol was used as the solvent in large excess with sodium hydroxide as the base. The mono-benzylation was afforded by quenching the mono-sodium salt of ethylene glycol with benzylbromide at 90°C for 3 hours to give **25** in 61% yield. Allylation of the other terminal alcohol was then performed in tetrahydrofuran with anhydrous basic conditions and allyl bromide to give **26** in reasonable yield. The

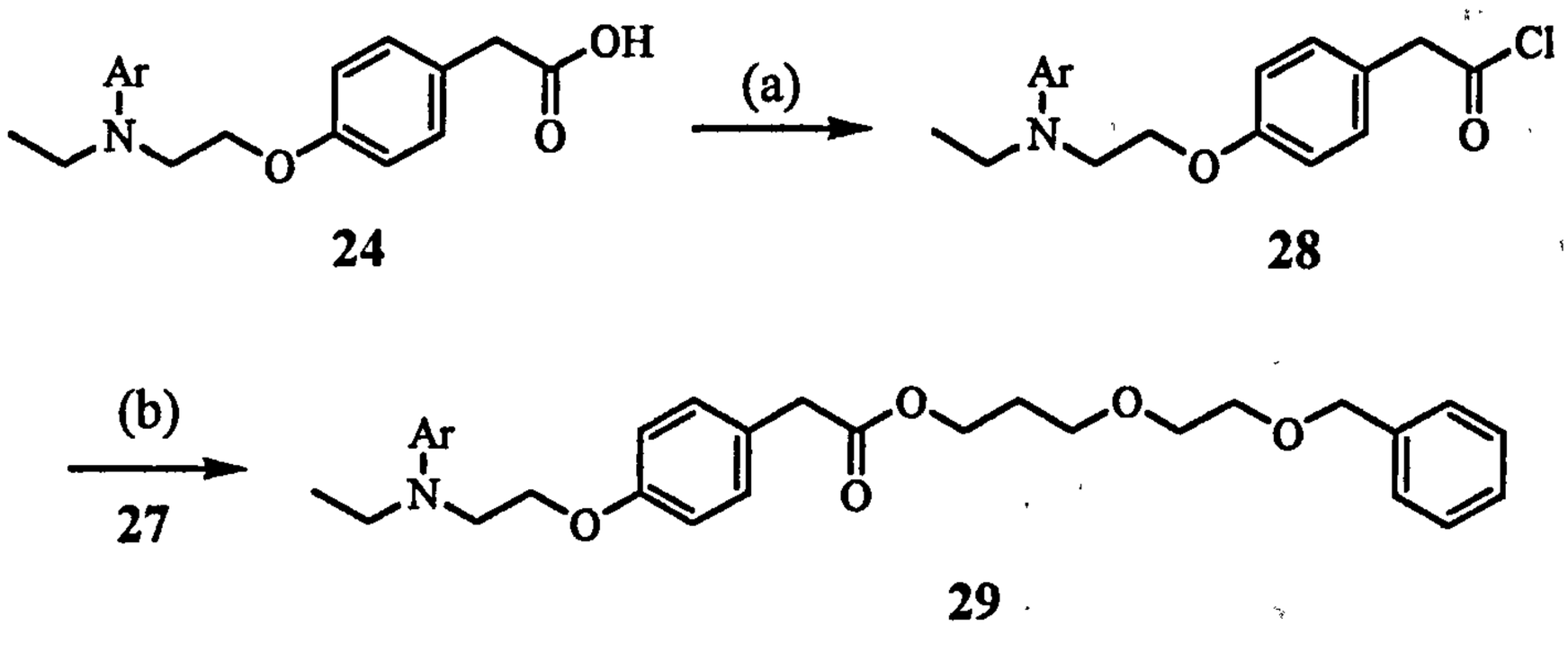
hydroboration was undertaken with borane dimethyl sulfide in toluene, and an oxidative work up with hydrogen peroxide in the presence of sodium hydroxide gave **27** in 45 % yield.



(a) Ethylene glycol (8 eq), sodium hydroxide (1 eq), molecular sieves, benzylbromide (1 eq), 90°C, 3 hours, 61%. (b) **25** (1.0 eq), sodium hydride (1.1 eq), allyl bromide (1.2 eq), tetrahydrofuran, room temperature, 4 hours, 78%. (c) **26** (1.0 eq), borane methyl sulfide (1.0 eq), room temperature 3 hours, methanol, hydrogen peroxide (1.2 eq), sodium hydroxide (2.5 eq), room temperature, 2 hours, 45%.

Scheme 2.12. The synthesis of the mono-benzylated linker **27**.

Coupling the mono-protected linker **27** to the acid derivative **24** of disperse red, was performed by forming the acid chloride of **24** *via* oxalyl chloride[59] in toluene, and then addition of **27** (Scheme 2.13).

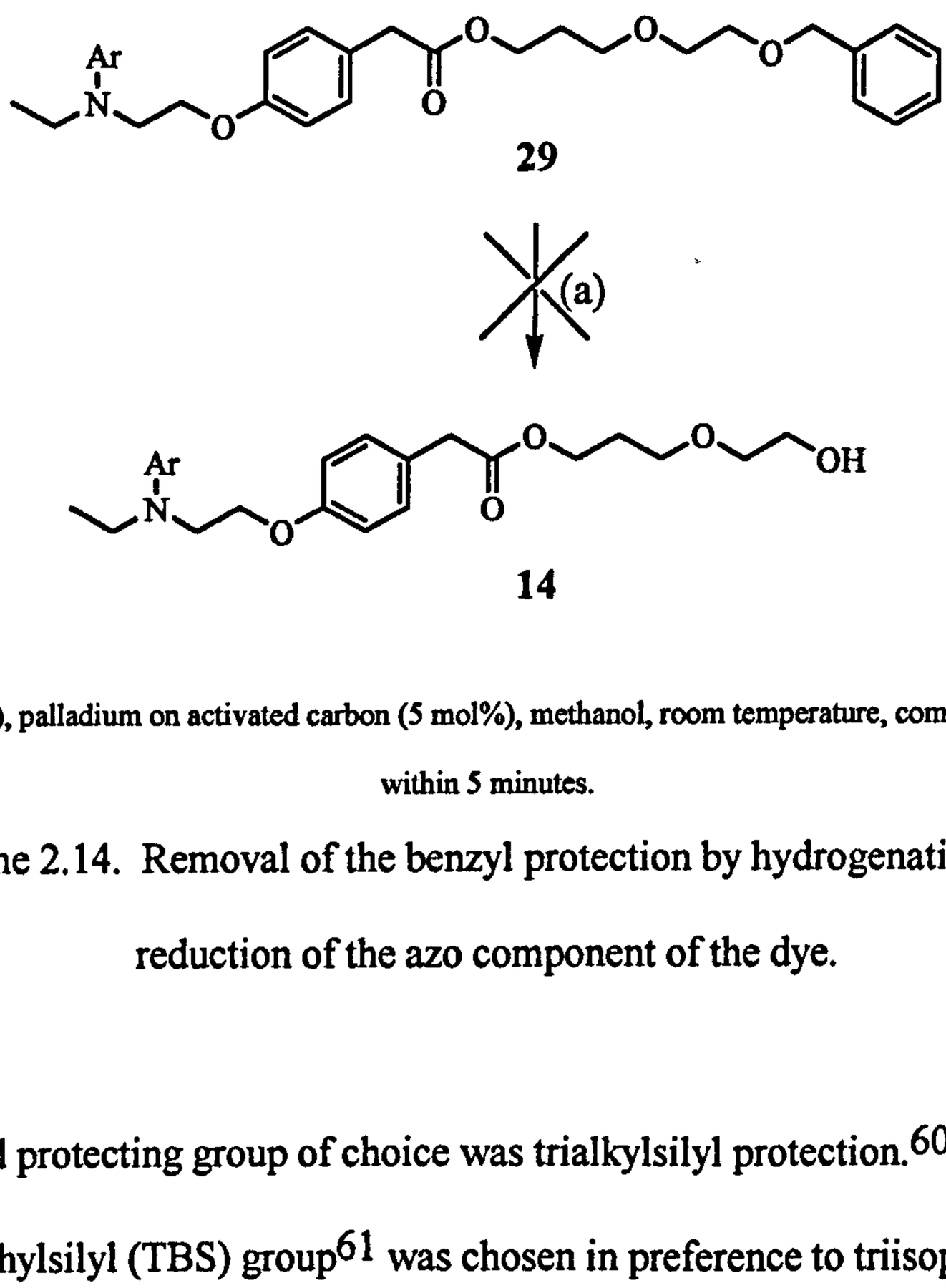


(a) **24** (1.0 eq), oxalyl chloride (1.0 eq), dimethylformamide (catalytic), toluene, room temperature, 4 hours, full conversion. (b) **27** (1.0 eq), triethylamine (2 eq), toluene, room temperature, 4 hours, 31%.

Scheme 2.13. Formation of **29** *via* the acid chloride of **24**.

The formation of the acid chloride could be monitored by observing the disappearance of **24** which was only sparingly soluble in toluene, and the evolution of carbon dioxide. The reaction was also followed by infra red spectroscopy; the carbonyl of the acid appeared at 1695 cm^{-1} and the carbonyl of the acid chloride appeared at 1765 cm^{-1}. The formation of the acid chloride was also catalysed by the addition of dimethylformamide (3 drops) and took about 2 hours. Unfortunately the yield of **29** *via* the acid chloride, was only 31% due to the formation of an unknown complex polar mixture. Therefore a second route to synthesise **29**, utilising the Mitsunobu alkylation, was investigated and a higher yield of 58% was achieved in which the dimerisation of disperse red was a minor competing reaction. Again the order of reagent addition was important and the acid derivative of disperse red **24**, had to be added last. The reaction was performed at room temperature and full conversion was achieved within 1 hour.

The final stage of the synthesis of the original dye-linker **14**, was the removal of the benzyl group (Scheme 2.14). This was performed by catalytic hydrogenation in the presence of (5 mol%) palladium on activated carbon. Unfortunately the dye-linker exhibited complete colour loss within 2 minutes. This was probably due to the reduction of the azo component which we had hoped would have been stabilised sufficiently *via* delocalisation. It might have been possible to apply a milder reducing system to effect selective deprotection of the benzyl group in the presence of the azo component, such as transfer hydrogenation. However, we rationalised that this might not have been a productive strategy and a different protecting group was sought.



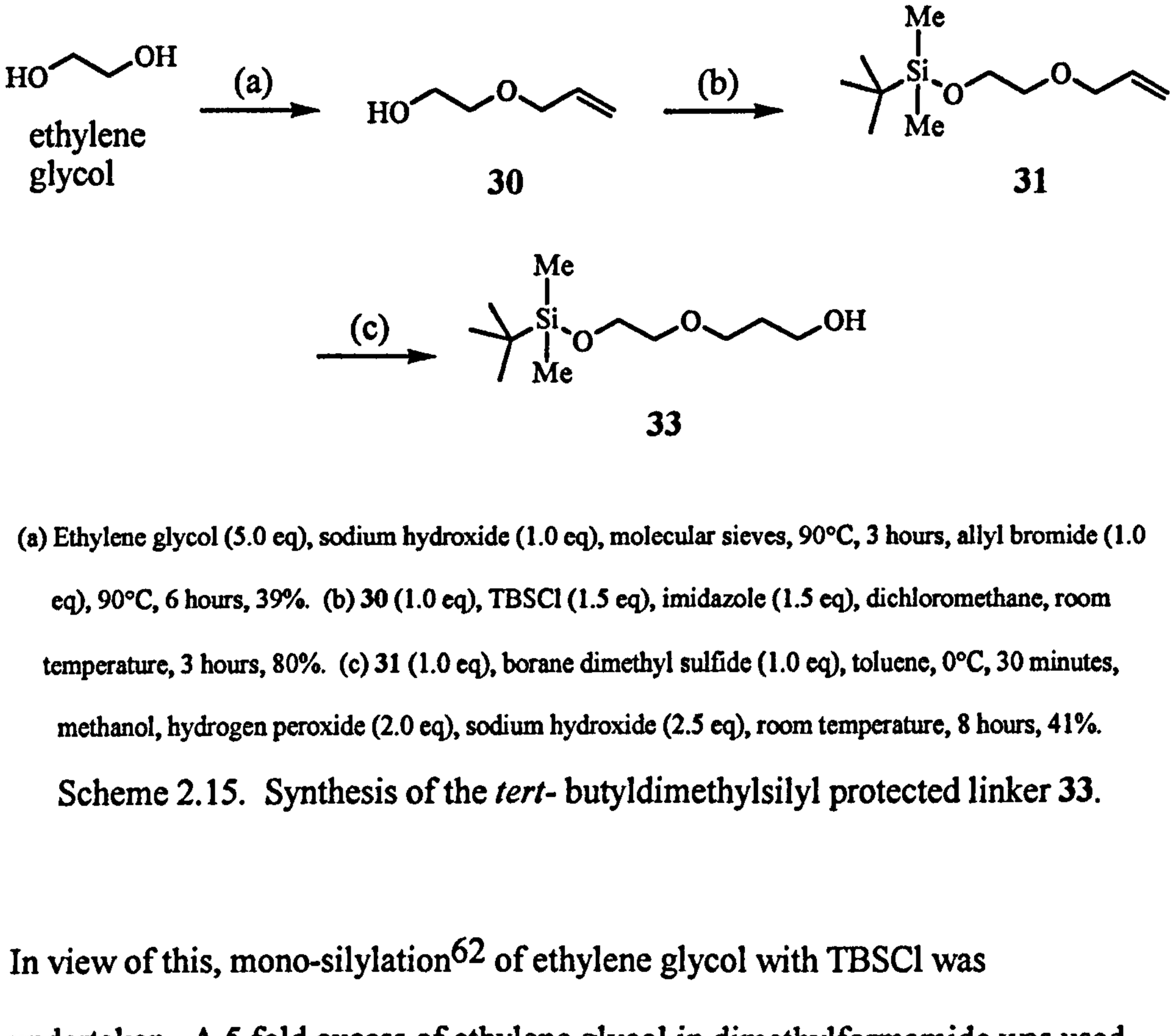
29 **14**

(a) 29 (1.0 eq), palladium on activated carbon (5 mol%), methanol, room temperature, complete colour loss within 5 minutes.

Scheme 2.14. Removal of the benzyl protection by hydrogenation gave reduction of the azo component of the dye.

The second protecting group of choice was trialkylsilyl protection.[60] The *tert*-butyldimethylsilyl (TBS) group[61] was chosen in preference to triisopropylsilyl

(TIPS) and *tert*-butyldiphenylsilyl (TBDPS) groups because it was more labile to deprotection conditions whilst still stable to flash column chromatography. Initially the synthesis of the TBS protected linker **33** shown in Scheme 2.15, was performed by allylation of ethylene glycol. The glycol was again used as the solvent in a large excess to avoid bis-allylation with sodium hydroxide as the base. Surprisingly a low yield of 2-allyloxyethanol **30** was obtained and the major product was the bis-allyl glycol. This was also observed during the synthesis of mono-allyl glycol substrates for vancomycin derivatives. It appears the mono-allyl products are more reactive to subsequent allylation than ethylene glycol. However, silylation of 2-allyloxyethanol **30** with *tert*- butyl dimethyl silyl chloride gave **31** in a reasonable yield of 80%.

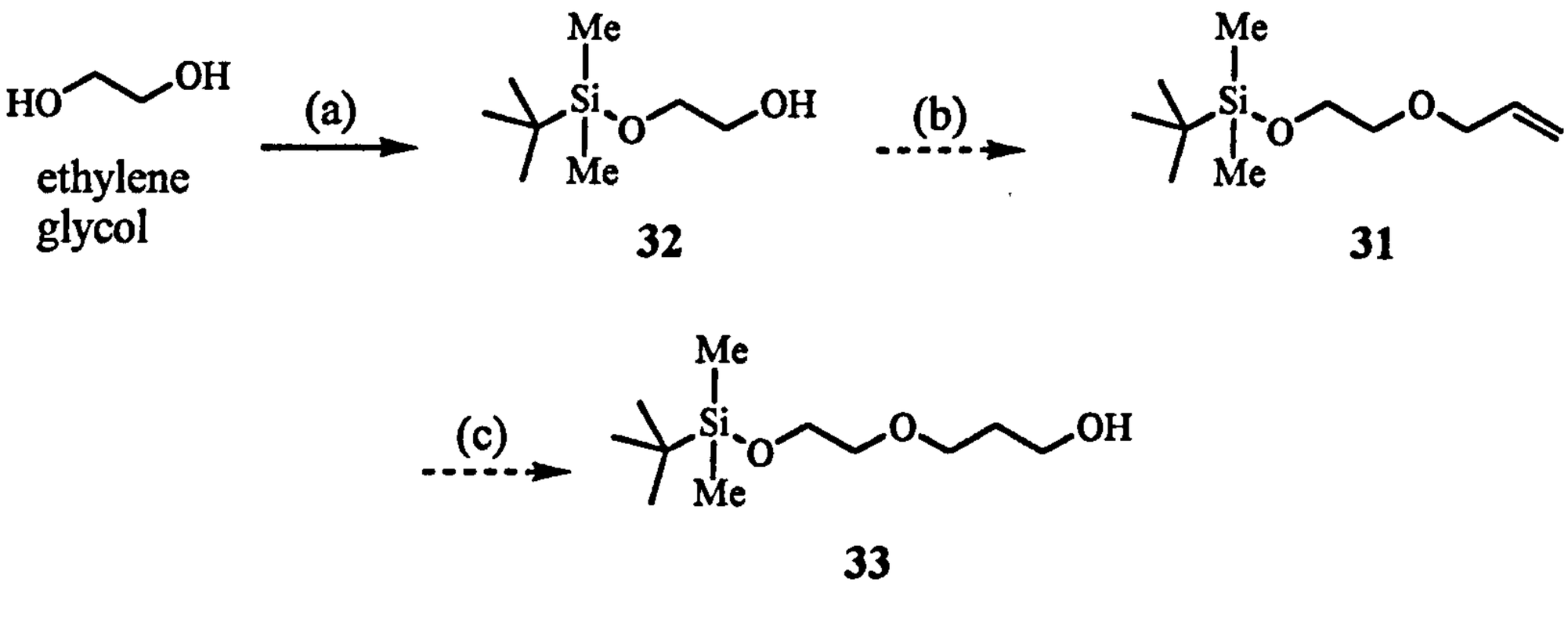


(a) Ethylene glycol (5.0 eq), sodium hydroxide (1.0 eq), molecular sieves, 90°C, 3 hours, allyl bromide (1.0 eq), 90°C, 6 hours, 39%. (b) **30** (1.0 eq), TBSCl (1.5 eq), imidazole (1.5 eq), dichloromethane, room temperature, 3 hours, 80%. (c) **31** (1.0 eq), borane dimethyl sulfide (1.0 eq), toluene, 0°C, 30 minutes, methanol, hydrogen peroxide (2.0 eq), sodium hydroxide (2.5 eq), room temperature, 8 hours, 41%.

Scheme 2.15. Synthesis of the *tert*- butyldimethylsilyl protected linker **33**.

In view of this, mono-silylation[62] of ethylene glycol with TBSCl was undertaken. A 5 fold excess of ethylene glycol in dimethylformamide was used

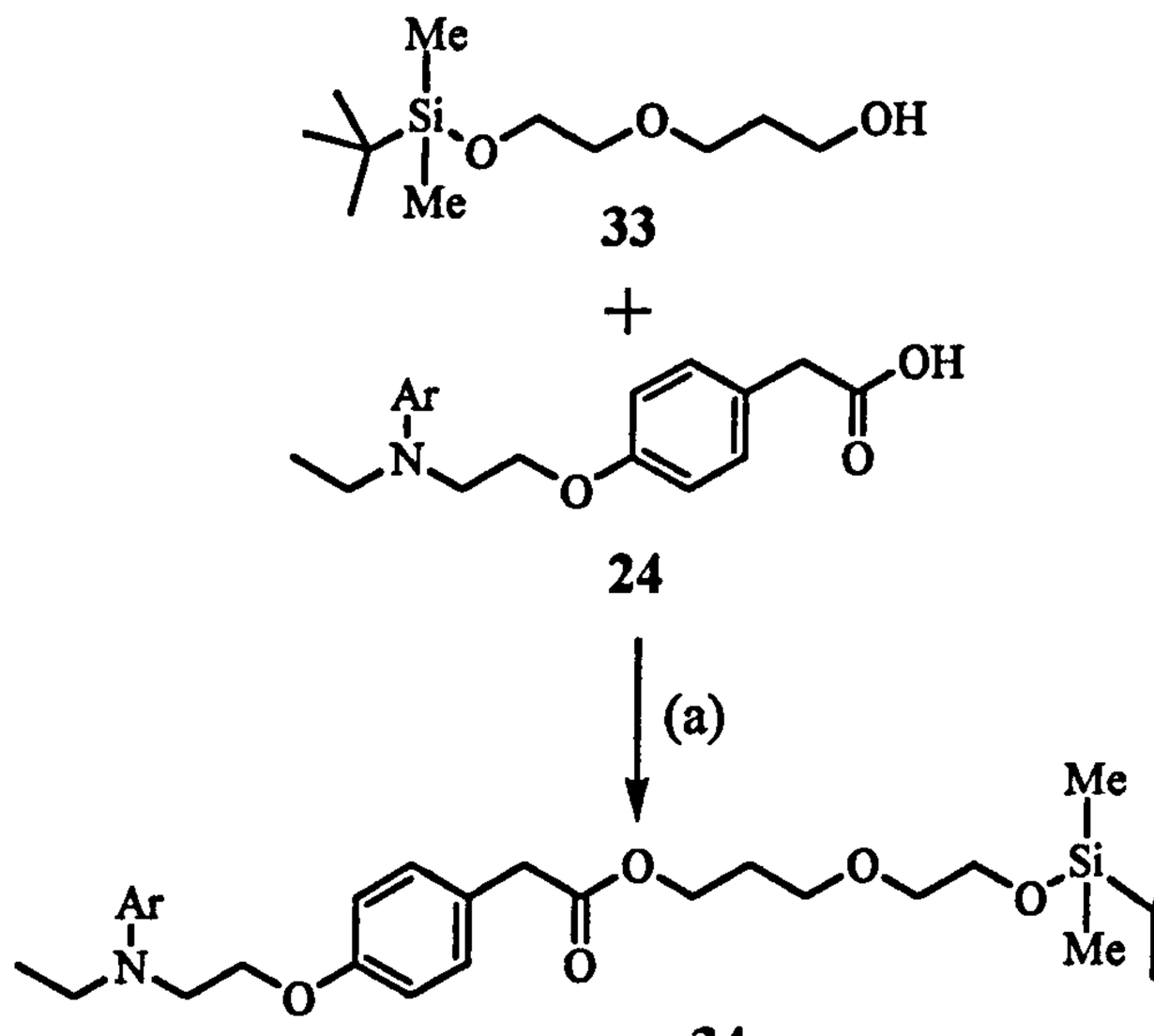
with imidazole as base, and mono-silylated ethylene glycol **32** was obtained in a reasonable yield of 74% (Scheme 2.16). Subsequent allylation of **32** with allyl bromide was not undertaken at this stage as a large amount of 2-allyloxyethanol **30** had already been synthesised. **31** was then subjected to hydroboration with borane dimethyl sulfide in toluene followed by an oxidative work up with sodium hydroxide to yield the TBS protected linker **33,** with a terminal hydroxy function.



(a) Ethylene glycol (5.0 eq), TBSCl (1.0 eq), imidazole (2.0 eq), dimethylformamide, room temperature, 12 hours, 74%. (b) Allylation with allyl bromide under anhydrous basic conditions. (c) Hydroboration with borane dimethyl sulfide followed by an oxidative work up with hydrogen peroxide.

Scheme 2.16. Plausible contingency should silylation be undertaken before allylation.

The coupling of **33** to **24** *via* Mitsunobu alkylation was then investigated and again it was found it was favourable to add the acid derivative of disperse red **24** last, although at a different temperature of 50°C (Scheme 2.17). The reaction mixture was stirred at this temperature for 5 hours and then at room temperature for two days. This afforded **34** in an excellent yield of 83% on a relatively large scale of 6 g.

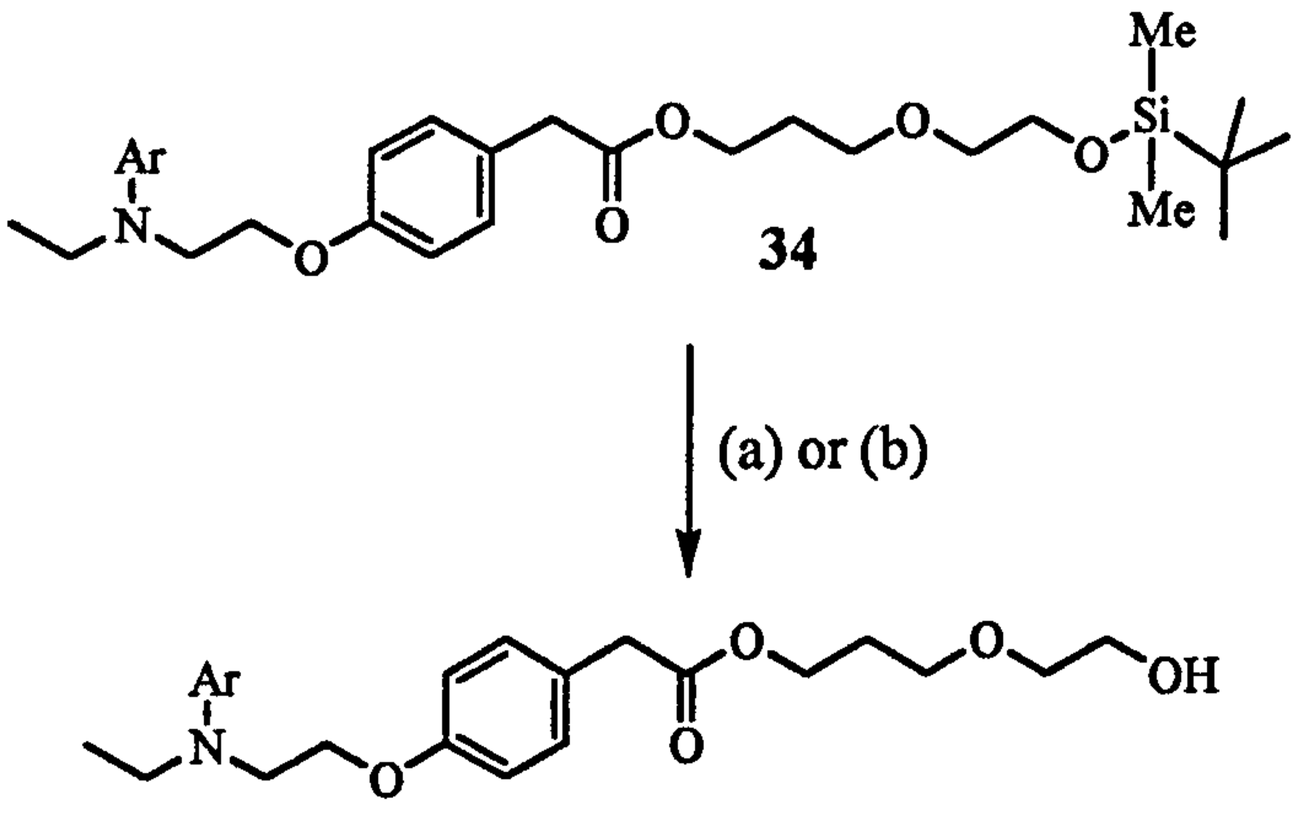


(a) **33** (1.0 eq), DEAD (1.0 eq), PPh_3 (1.0 eq), **24** (0.67 eq), toluene, 50°C 5 hours, room temperature 2 days, 83%.

Scheme 2.17. Coupling the silyl protected linker **33** to the acid derivative of disperse red **24** *via* Mitsunobu alkylation.

The final deprotection of **34** was initially performed using TBAF in tetrahydrofuran.[61] On a small scale a low yield of 34% was realised however, unfortunately on a larger scale the yield was very low, 9%, and a complex mixture of polar compounds was formed. This may be due to **34** being unstable to the basic conditions of TBAF although it was shown previously that disperse

red itself was relatively stable to these conditions. In view of this we decided to effect the silyl deprotection with aqueous hydrogen fluoride (HF) in acetonitrile at room temperature.[63,64] Complete conversion was observed within 90 minutes to give the unprotected dye-linker **14** in a reasonable yield of 92% (Scheme 2.18).



original dye linker **14**

(a) **34** (1.0 eq), TBAF.3H_2O (1.1 eq), tetrahydrofuran, 40°C, 2 hours, 34%. (b) **34** (1.0 eq), 40% aqueous HF (5.0 eq), acetonitrile, room temperature, 90 minutes, 92%

Scheme 2.18. Silyl deprotection was cleanly afforded with aqueous HF.

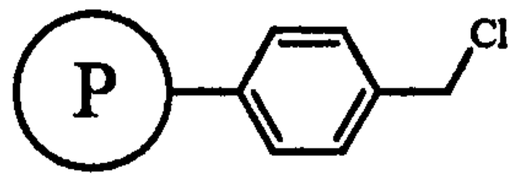
2.1.2 Polymer Support

A variety of polymer supports were commercially available and physical properties in which we were interested included; the polymer's ability to swell in a range of solvents, the level of loading and resistance to abrasion.. The choice of polymer support also depended on one of two general strategies for synthesising peptides on polymer support. These were the 9-fluorenylmethoxycarbonyl

(Fmoc) and *tert*- butoxycarbonyl (Boc) N-α-methods which will be discussed in more detail below. The Fmoc strategy was eventually chosen because it was a milder and more reliable method. There was also a significantly larger range of polymer supports available for the Fmoc method than the Boc strategy.

Merrifield resin[65] was the original resin developed for solid phase peptide synthesis (SPPS) and consists of polystyrene beads functionalised with chloromethyl groups onto which the first amino acid of the peptide is attached (Figure 2.1). This resin is used to synthesise peptides *via* the Boc strategy.

Three resins that have been developed to increase the level of loading that can be achieved on a bead, and increase their swelling abilities to a variety of solvents allowing active sites to be less hindered to reagents, are: Tentagel, Kieselguhr and PEGA gel resins.[66-68]



Merrified resin. Polystyrene beads functionalised with chloromethyl groups

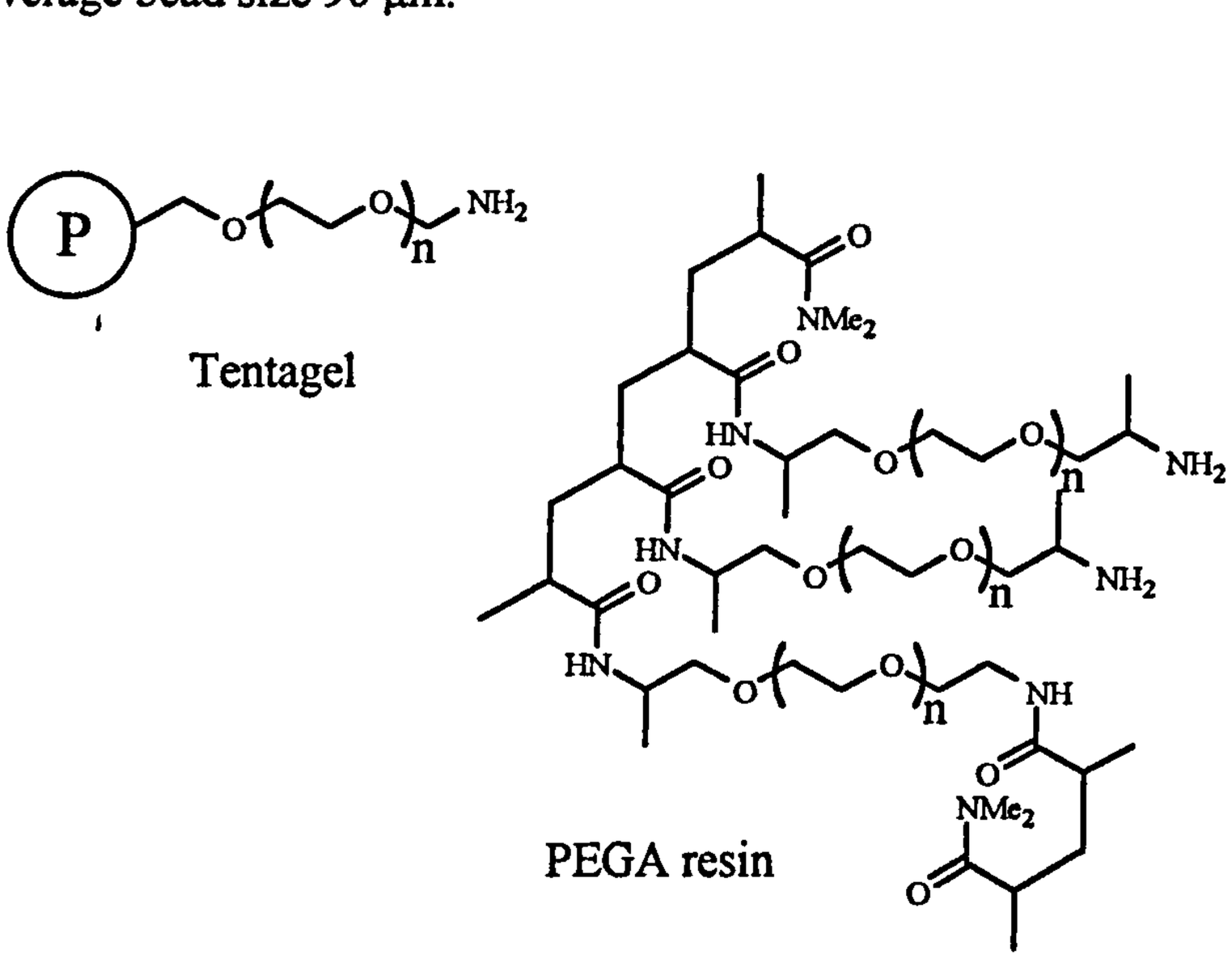
Figure 2.1. Merrifield resin.

Tentagel resin[67] consists of hydroxyethyl polystyrene beads onto which polyethylene glycol (PEG) chains of molecular weight 3000 – 4000 Da, have been grafted (Scheme 2.19). The long PEG chains give Tentagel the favourable physicochemical properties of being able to swell in a wide range of solvents from toluene to water, a high loading and improved chemical efficiency. Tentagel also has a reasonable resistance to mechanical abrasion.

Kieselguhr resins also have excellent swelling capabilities due to their high permeability to macromolecules. However Kieselguhr resin suffers attrition in stirred reactions and is more suitable for use in packed beds.

PEGA resins shown in Scheme 2.19, consist of 2-acrylamidoprop-1-yl-(2-aminoprop-1-yl) polyethylene glycol$_{800}$ and dimethylacylamide cross linked with bis 2-acrylamidoprop-1-yl polyethylene glycol$_{800}$. These supports swell extensively in a wide range of solvents and are freely permeable to macromolecules up to 35 KDa, making them well suited for the preparation of peptide libraries and on-resin enzyme assays.

In view of this we decided to use the cheaper Tentagel resin as it had the physical properties we required and the systems to be investigated were relatively small (~1-2 kDa). The average loading of a Tentagel resin was 0.15 – 0.30 mmol/g and the average bead size 90 μm.



Scheme 2.19. Tentagel and PEGA resin both utilise long ethylene glycol chains.

2.1.3 Attaching Disperse Red and Dye-Linker **14** to Tentagel

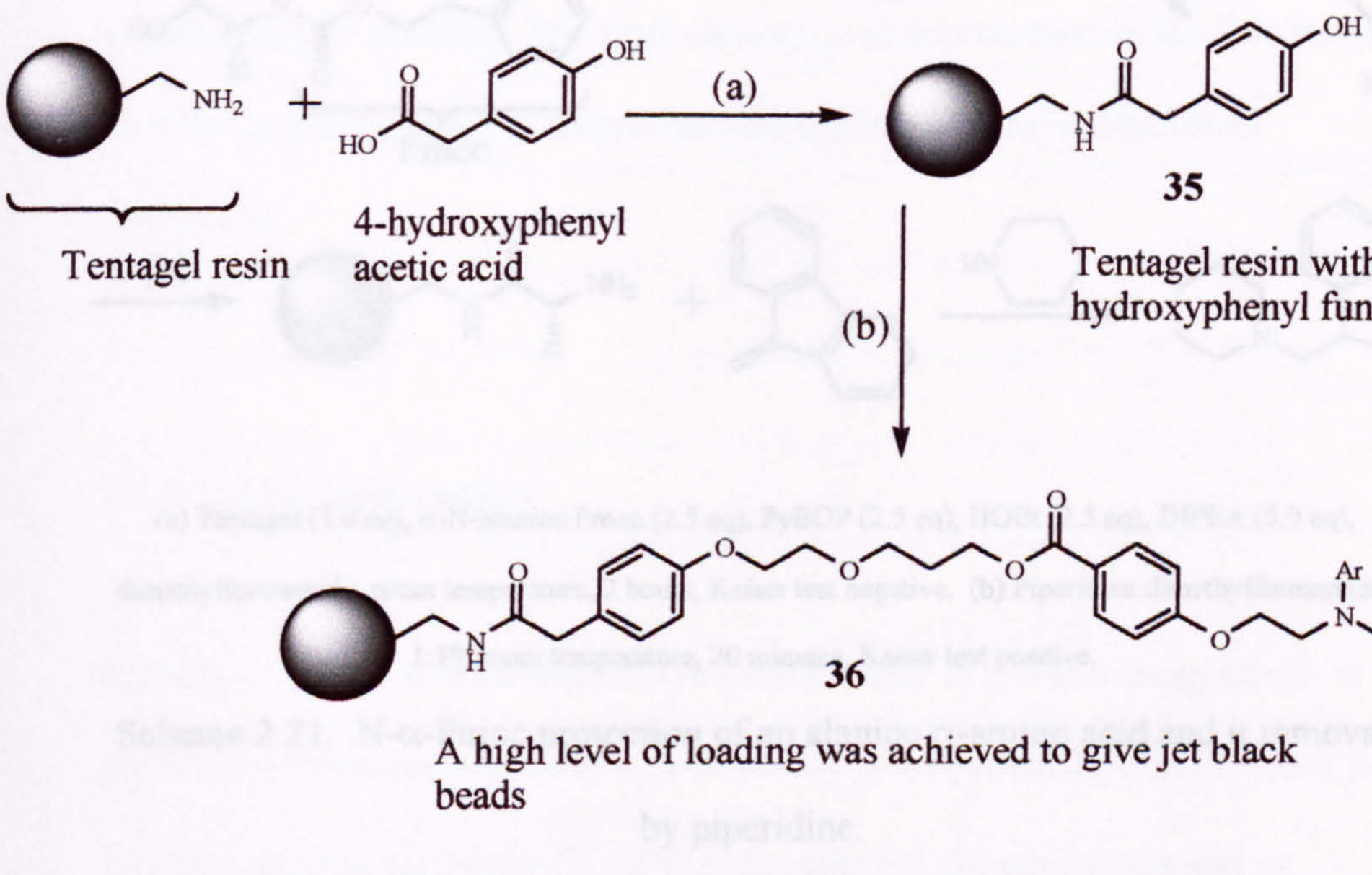
After choosing to manually synthesise our sixteen unit peptide, we decided it would be prudent to investigate whether it was possible to directly attach disperse red or the original dye-linker **14** to the same polymer support. If difficulties were experienced during simpler coupling to the polymer support, undertaking the same coupling technique to attach the dye-linker to the terminal end of a large and sterically hindered supported peptide, might also be difficult. We also wished to investigate if any physical staining of the resin occurred. The synthetic route chosen involved functionalising the free amino group of Tentagel resin with 4-hydroxyphenylacetic acid to give **35** and then coupling disperse red and the dye-linker **14** *via* Mitsunobu alkylation (Scheme 2.20). Tentagel resin was readily functionalised with 4-hydroxyphenylacetic acid in dimethylformamide and full conversion was observed within 1 hour at room temperature. The coupling agent, PyBOP, will be discussed in more detail below. The method of analysing the conversion *via* the Kaiser test, will also be discussed below.

After the Tentagel resin functionality had been changed from a free amino to a free hydroxyphenyl group **35**, it was found that the best conditions to couple both disperse red and the dye-linker **14**, were tetrahydrofuran as solvent at room temperature in the presence of triethylamine which had been shown by Richter *et al.*[69] to assist Mitsunobu reactions on polymer resins. It was also shown that it was important to use a concentrated reaction mixture and to stir the resin carefully to avoid the beads from disintegrating. Relying on diffusion only gave partial coupling to surfaces of resin readily exposed to the solution. Upon a high

level of loading the beads were converted from a pale yellow coloration to an extremely dark red, when solvated in methanol, and jet black when dry.

To investigate the possibility of physical adsorption, the resin **35** was subjected to the same Mitsunobu alkylation reaction conditions except where one of the reagents, DEAD, was excluded. The resin did acquire a slight light red coloration, however this was easily removed by light washing in methanol to give a resin that had a very light pink colour.

It was also demonstrated that no coupling occurred directly between amino functionalised Tentagel resin and the dye-linker under the optimum Mitsunobu alkylation conditions. Again a resin with a very light pink coloration was obtained.



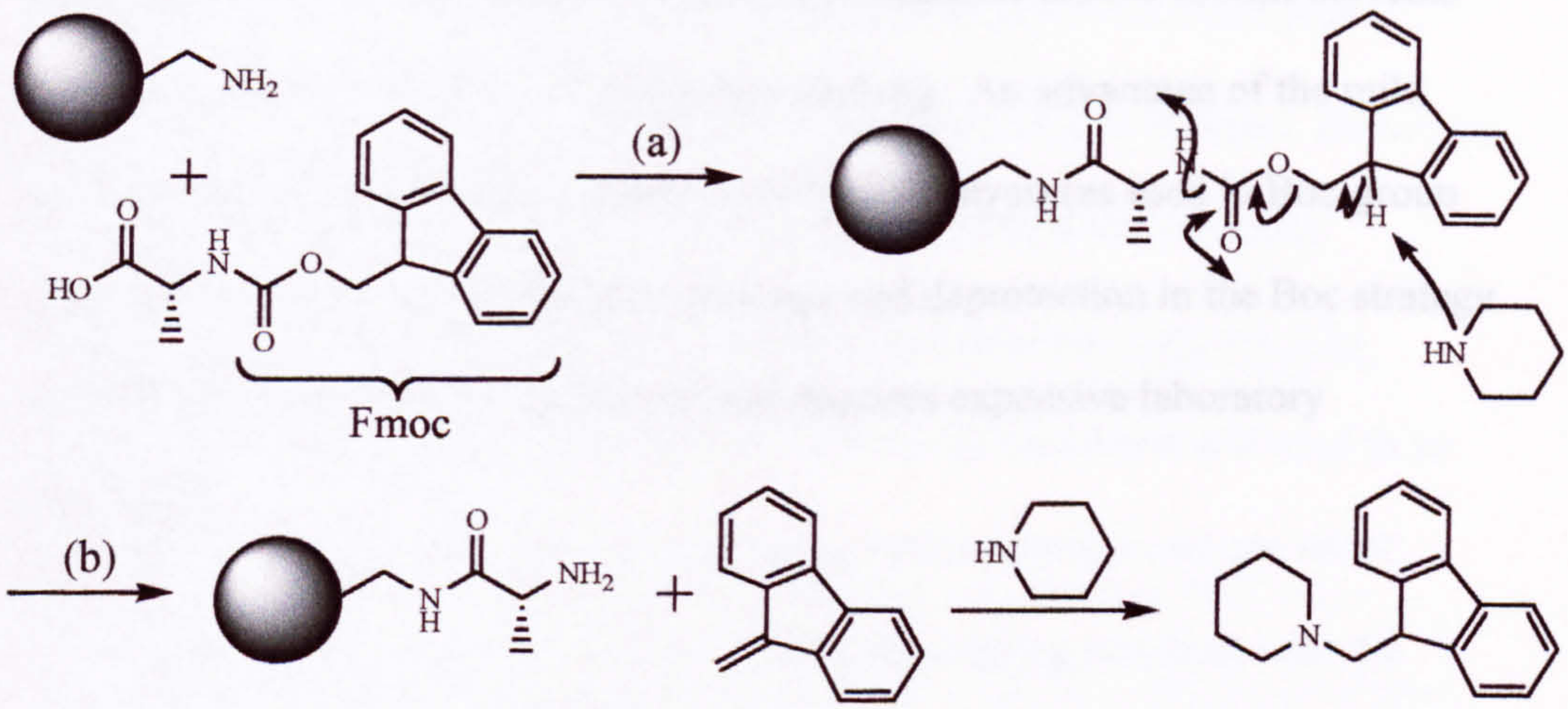
(a) Tentagel (1.0 eq), 4-hydroxyphenylacetic acid (2.5 eq), PyBOP (2.5 eq), HOBt (2.5 eq), DIPEA (5.0 eq), dimethylformamide, room temperature, 2 hours, Kaiser test negative. (b) **35** (1.0 eq), DEAD (2.0 eq), PPh_3 (2.0 eq), **14** (2.5 eq), triethylamine (5.0 eq), tetrahydrofuran, room temperature, 1 hour.

Scheme 2.20. Attaching the original dye-linker **14** to Tentagel.

2.1.4 Synthesis of The Sixteen Residue Peptide

2.1.4.1 The Fmoc Strategy

The SPPS involved the sequential addition of α-amino acid and side chain protected α-amino acid residues to an insoluble polymeric support. The two general strategies for applying SPPS required the use of the base-labile Fmoc group and acid-labile Boc group, for N-α-protection. The Fmoc group and its removal are shown in Scheme 2.21.



(a) Tentagel (1.0 eq), α-N-alanine Fmoc (2.5 eq), PyBOP (2.5 eq), HOBt (2.5 eq), DIPEA (5.0 eq), dimethylformamide, room temperature, 2 hours, Kaiser test negative. (b) Piperidine:dimethylformamide, 1:19, room temperature, 20 minutes, Kaiser test positive.

Scheme 2.21. N-α-Fmoc protection of an alanine α-amino acid and it removal by piperidine.

After removal of the protecting group, the next protected amino acid is added using either a coupling reagent or pre-activated protected amino acid derivative. The resulting peptide is attached to the resin *via* a linker, and may be cleaved to yield a peptide acid or amide, depending on the linking agent used. Side chain

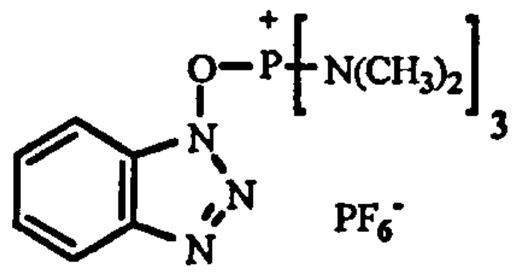
protecting groups are often chosen so as to be cleaved simultaneously with detachment of the peptide from the resin.

Cleavage of the Boc protecting group is achieved by trifluoroacetic acid (TFA) and the Fmoc protecting group by piperidine. With regard to the Boc strategy, final cleavage of the peptidyl resin and side chain deprotection requires acidic conditions, such as HF or trifluoromethanesulfonic acid (TFMSA). For the Fmoc strategy, the final cleavage and deprotection is effected with trifluoroacetic acid (TFA). Dichloromethane and dimethylformamide are the routine solvents used for resin deprotection, coupling and washing. An advantage of the mild base process of Fmoc SPPS is repetitive TFA hydrolysis (as used in Boc-group deprotection) is avoided. The final cleavage and deprotection in the Boc strategy with HF is also potentially dangerous and requires expensive laboratory equipment.

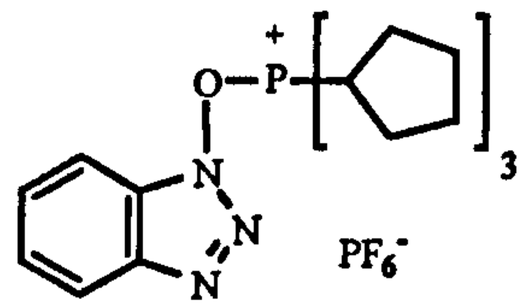
2.1.4.2 Coupling Reagents

Carbodiimides have been some of the most popular *in situ* activating agents in peptide synthesis and an example is dicyclohexylcarbodiimide (DCC) which was first described in the 1950s.[70] DCC is an effective coupling agent and its activity can be increased by the addition of N-hydroxybenzotriazole (HOBt). However, a major drawback of DCC is that is it generates dicyclohexylurea, which is insoluble in DCM and can be a cause of contamination.

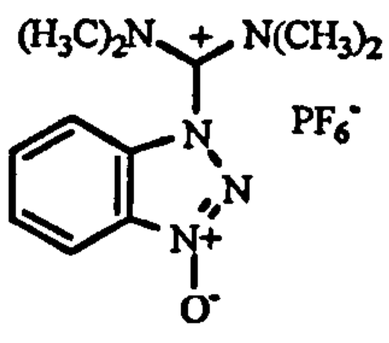
Several other coupling reagents have been designed, some of which are shown in Figure 2.2. The phosphonium and uronium salts are commonly employed due to their ease of use and high efficiency of forming activated esters in the presence of HOBt[71] and a tertiary base such as diisopropylethyl amine (DIPEA). Side reactions and epimerisation of chiral centers are also reduced. However, care should be taken when using benzotriazole-1yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP)[72] as a side product is hexamethylphosphotriamide (HMPA) which is thought to be a carcinogen. Benzotriazole-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyBOP)[73] can be used in place of BOP without loss of performance. However, the uronium salts 2-(N-benzotriazole-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate (HBTU)[74,75] and 2-(N-benzotriazole-1-yl)-1,1,3,3,-tetramethyluronium tetrafluoroborate (TBTU) can be troublesome if used in an excess to the amino acid, since capping of the amino terminal end can occur through guanidine formation. The phosphonium coupling reagents, with the exception of bromo-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBroP)[76] in dimethylformamide, do not give rise to such by-products. However, PyBroP is particularly useful for the coupling of N-methyl amino acids which can be a difficult and slow process. PyBroP allows efficient, fast and enantiomerisation free coupling.



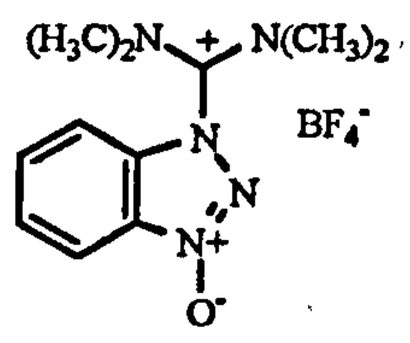
BOP



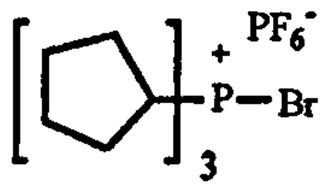
PyBOP



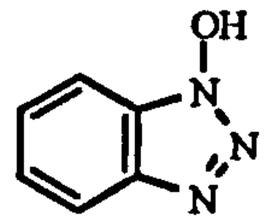
HBTU



TBTU



PyBroP



HOBt

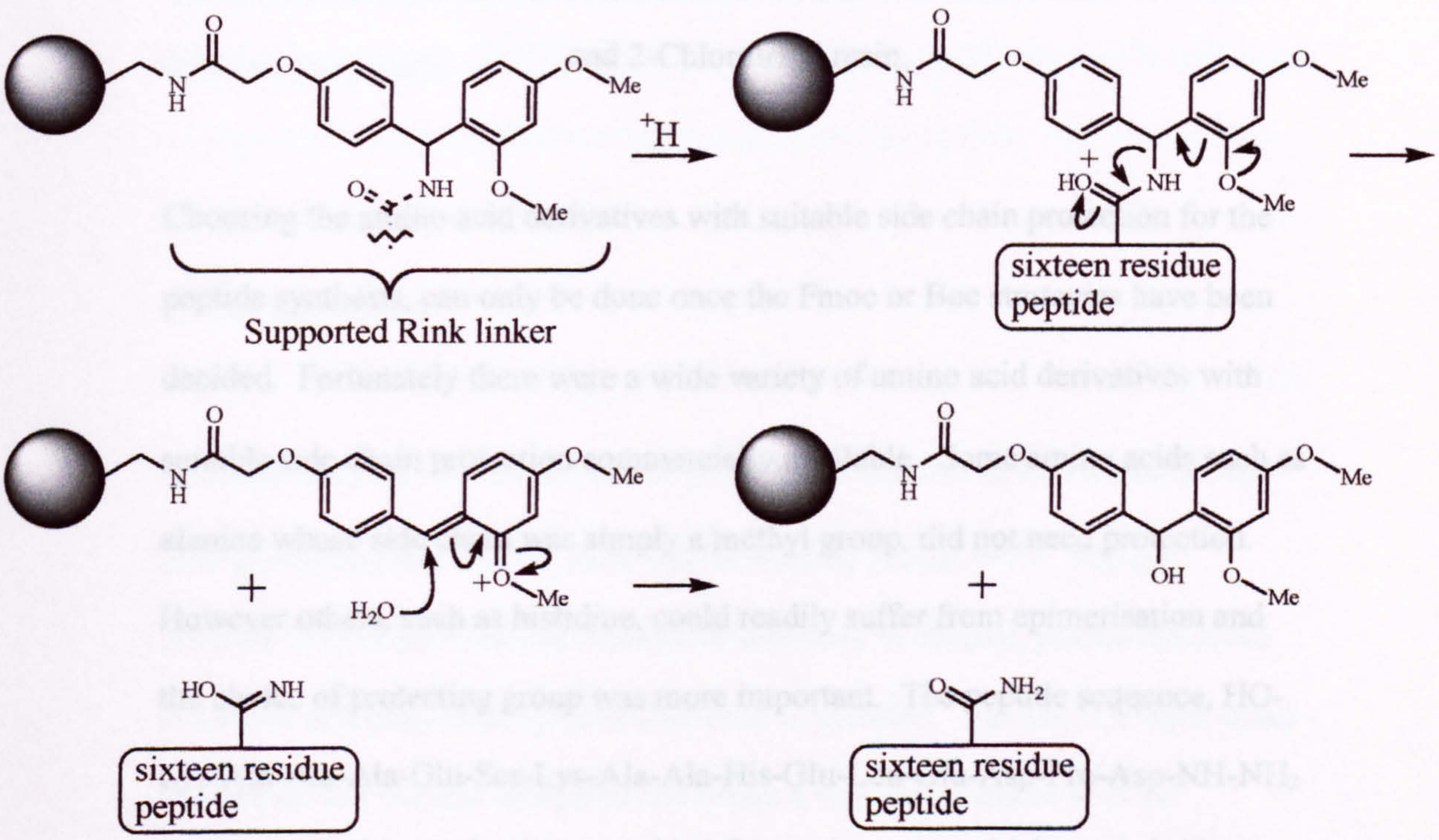
Figure 2.2. Six common coupling reagents used during SPPS.

In view that PyBOP was an efficient coupling reagent whereas BOP was a possible source of a carcinogen, the uronium salts could be problematic if used in excess and that PyBroP was expensive, PyBOP was the coupling reagent chosen to synthesise the sixteen residue peptide.

2.1.4.3 The Linker and Amino Acid Protection Groups

As discussed in the previous chapter we wished to use a polymer support of which 10% contained an acid labile linker[77] before the peptide. This would allow

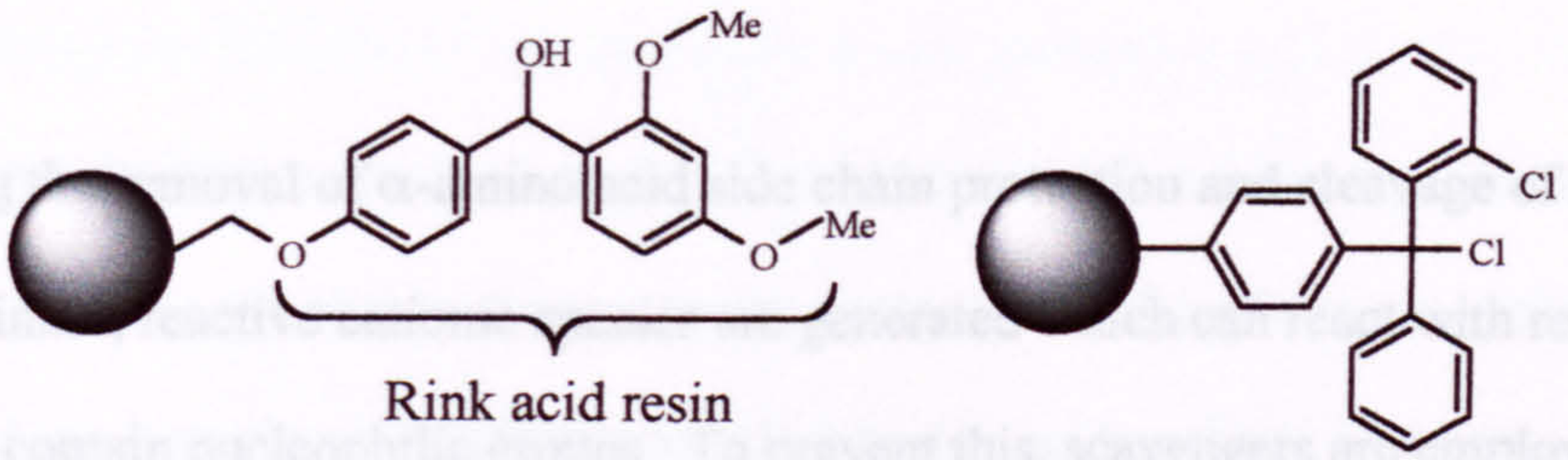
10% of the peptide to be cleaved from the support during deprotection of the peptide thus giving a means for analysis *via* mass spectrometry. An extensive range of resins based on the acid labile Rink amide linker[78] for the production of peptide amides by Fmoc synthesis were commercially available. This included Tentagel which was supplied with the Rink amide linker attached, but unprotected allowing its direct use alongside the normal Tentagel resin. The structure of the supported Rink amide linker and its mechanism of cleavage are shown in Scheme 2.22.



Scheme 2.22. Acid catalysed cleavage of the Rink linker.

Other supported linkers were available such as Rink acid resin and 2-chlorotritylchloride resin (Figure 2.3). However, these linkers were highly labile to acid and designed to be cleaved in conditions mild enough not to affect

deprotection of the peptide's amino acid side chains. This allows a possible route for the synthesis of a peptide that is still protected.



Rink acid resin

2-Chlorotrityl resin

Figure 2.3. Two supported linkers that are highly acid labile are Rink acid resin and 2-Chlorotrityl resin.

Choosing the amino acid derivatives with suitable side chain protection for the peptide synthesis, can only be done once the Fmoc or Boc strategies have been decided. Fortunately there were a wide variety of amino acid derivatives with suitable side chain protection commercially available. Some amino acids such as alanine whose side chain was simply a methyl group, did not need protection. However others, such as histidine, could readily suffer from epimerisation and the choice of protecting group was more important. The peptide sequence, HO-Lys-Ala-Ala-Ala-Glu-Ser-Lys-Ala-Ala-His-Glu-Leu-Glu-Asp-Pro-Asp-NH-NH_2 that we wished to synthesise, contained five amino acids which needed side chain protection. These and the protecting groups for the side chains selected on the basis of known stability and deprotection protocol, were:

a) *tert*-Butyl for aspartic acid, glutamic acid and serine.

b) *tert*-Butyl carbamate for lysine.

c) Trityl for histidine.

During the removal of α-amino acid side chain protection and cleavage of the Rink linker, reactive cationic species are generated which can react with residues which contain nucleophilic groups. To prevent this, scavengers are employed to react with these ions. A scavenger used in the Fmoc strategy is triisopropylsilane (TIS) which is known to be a hydride source in TFA[79] and this was used below.

2.1.4.4 Resin Tests

A widely used qualitative test for the presence or absence of free amino groups during coupling and deprotection, was devised by Kaiser.[80,81] The test involves 3 different solutions: ninhydrin dissolved in ethanol, phenol dissolved in ethanol and potassium cyanide dissolved in pyridine. The test is simple, quick and similar to a normal ninhydrin test where a free amine reacts with the ninhydrin upon heating. A blue solution and a resin that is also stained blue, is obtained if an amine is present. The phenol is present to help swell the resin and the potassium cyanide is present to sensitise the test. The procedure involves subjecting a few milligrams of the resin in a test tube, to 3 drops of each solution and the green suspension is heated for 2-3 minutes at 80°C in a water bath. Any colour changes are recorded. An intermediate Kaiser test is indicated by the

solution remaining a green colour (no colour change) but the resin is dyed red when viewed under a microscope. A negative Kaiser test is indicated when both the solution and resin remain the same colour.

Although the Kaiser test is general, it should be noted that some deprotected amino acids do not always show a positive test. Examples of this are serine, asparagine, aspartic acid and the secondary amino acid proline. Strongly aggregated sequences can also give false negative tests. Therefore, it is important to be careful when assessing the extent of deprotection.

Other resin tests available include 2,4,6-trinitrobenzenesulfonic acid (TNBS) and *p*-chloroanil where p-chloroanil is recommended for proline. The two tests are described below;

TNBS resin test. A few milligrams of resin are suspended in dimethylformamide to which three drops of diisopropylethyl amine (DIPEA):dimethylformamide, 1:9 and three drops of TNBS:dimethylformamide, 1:99 are added. The suspension is left to stand at room temperature for 5 minutes after which the resin is washed with dimethylformamide. A positive test (indicating the presence of free amino groups) is indicated by the resin being stained red. However, if this test is required to prove a coupling stage has gone to completion (a negative test is required), it is important not to apply heat as the Fmoc protecting group can be cleaved. Subsequently a false positive test will result.

p-Chloroanil test. A few milligrams of resin are suspended in dimethylformamide (0.5 ml) to which three drops of

acetaldehyde:dimethylformamide, 1:49 and three drops of *p*-chloroanil:dimethylformamide, 1:49 are added. The suspension is allowed to stand at room temperature for 10 minutes after which time a positive test (indicating the presence of free secondary amino groups) is indicated by the resin being stained blue.

2.1.4.5 SPPS Reaction Vessel

The simple peptide vessel shown in Figure 2.4 was constructed from a sintered glass funnel. It has a three way teflon tap at the bottom and a B24 ground glass joint at the top which could be connected to a nitrogen bubbler. Agitation of the reaction mixture within the peptide vessel was achieved by gently passing nitrogen through the sinter *via* the three way tap. The rate of nitrogen flow was observed by use of the nitrogen bubbler. After the reaction had gone to completion, the reaction solution could then be removed under reduced pressure *via* the three way tap. Washing the resin with various solvents was undertaken in the same manner which meant the resin was continuously contained within the peptide vessel. Small samples could be removed from the bulk with a long glass pasteur pipette.

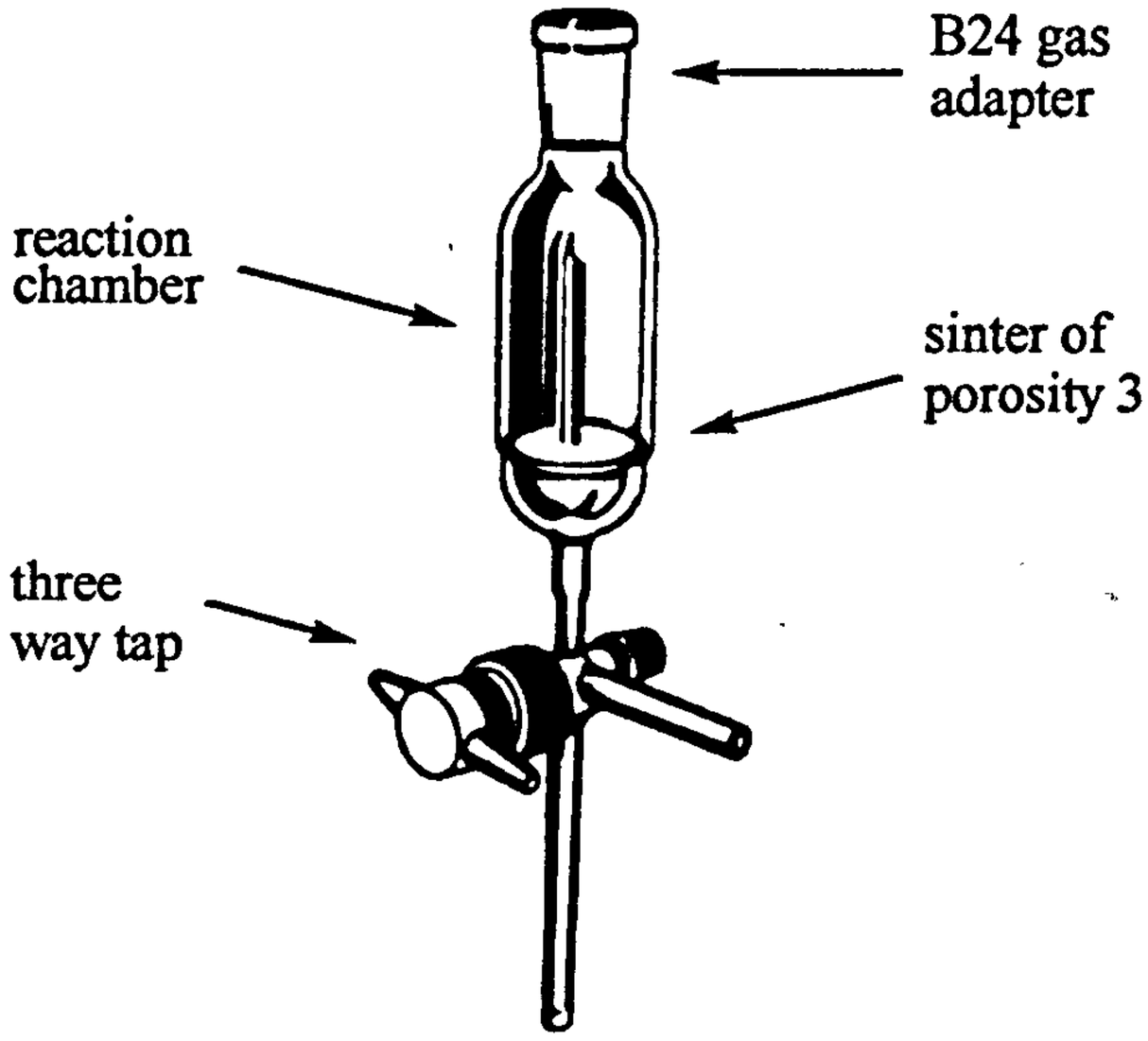
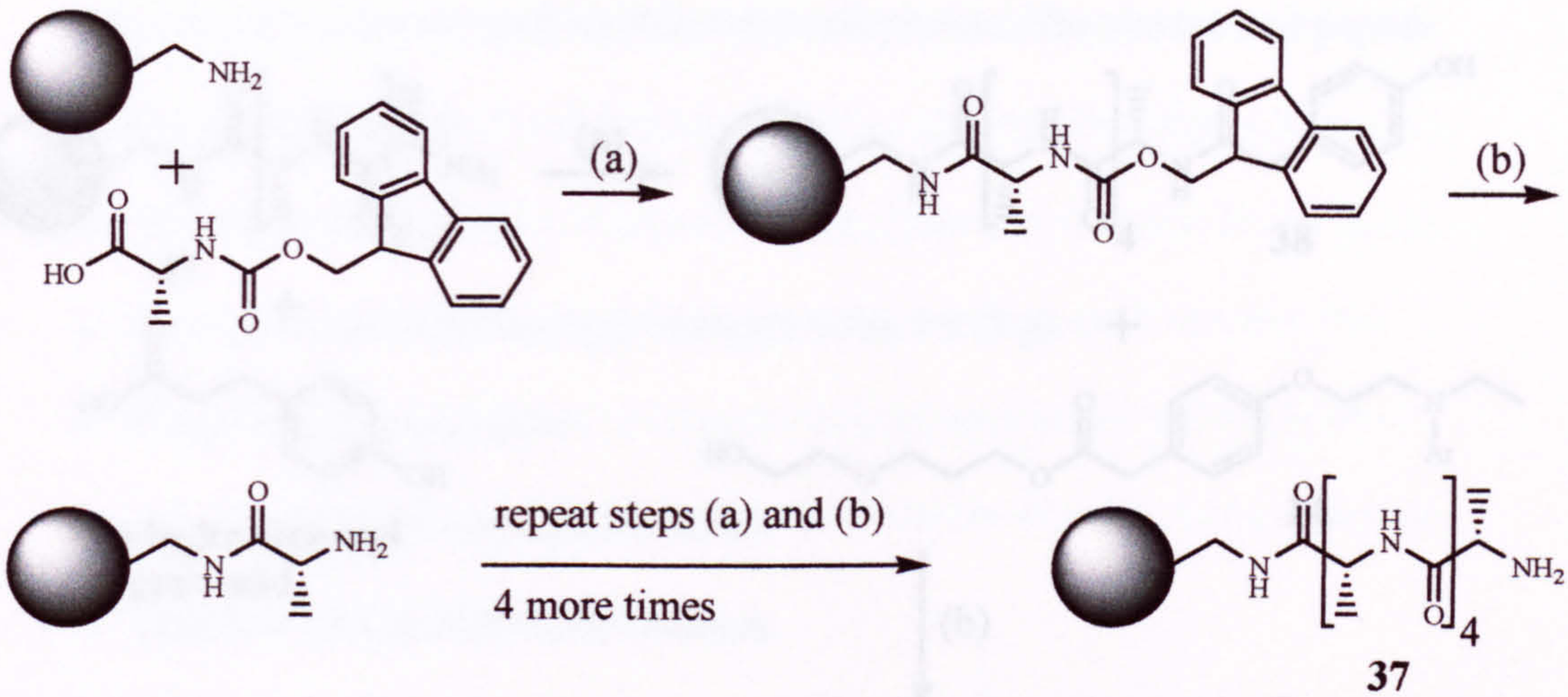


Figure 2.4. The peptide reaction vessel. The reaction mixture could be subjected to a supply of nitrogen or reduced pressure *via* the three way tap.

2.1.4.6 Synthesis of a Pentapeptide

A short supported pentapeptide HO-Ala-Ala-Ala-Ala-Ala-NH_2 was initially synthesised on Tentagel in the peptide vessel to gain experience of using the reaction vessel and the general procedures needed to synthesise the larger sixteen residue peptide. Although the dye-linker had been shown to couple to Tentagel functionalised with 4-hydroxyphenylacetic acid *via* Mitsunobu alkylation, we decided it would also be prudent to show the same could be done with the supported pentapeptide.

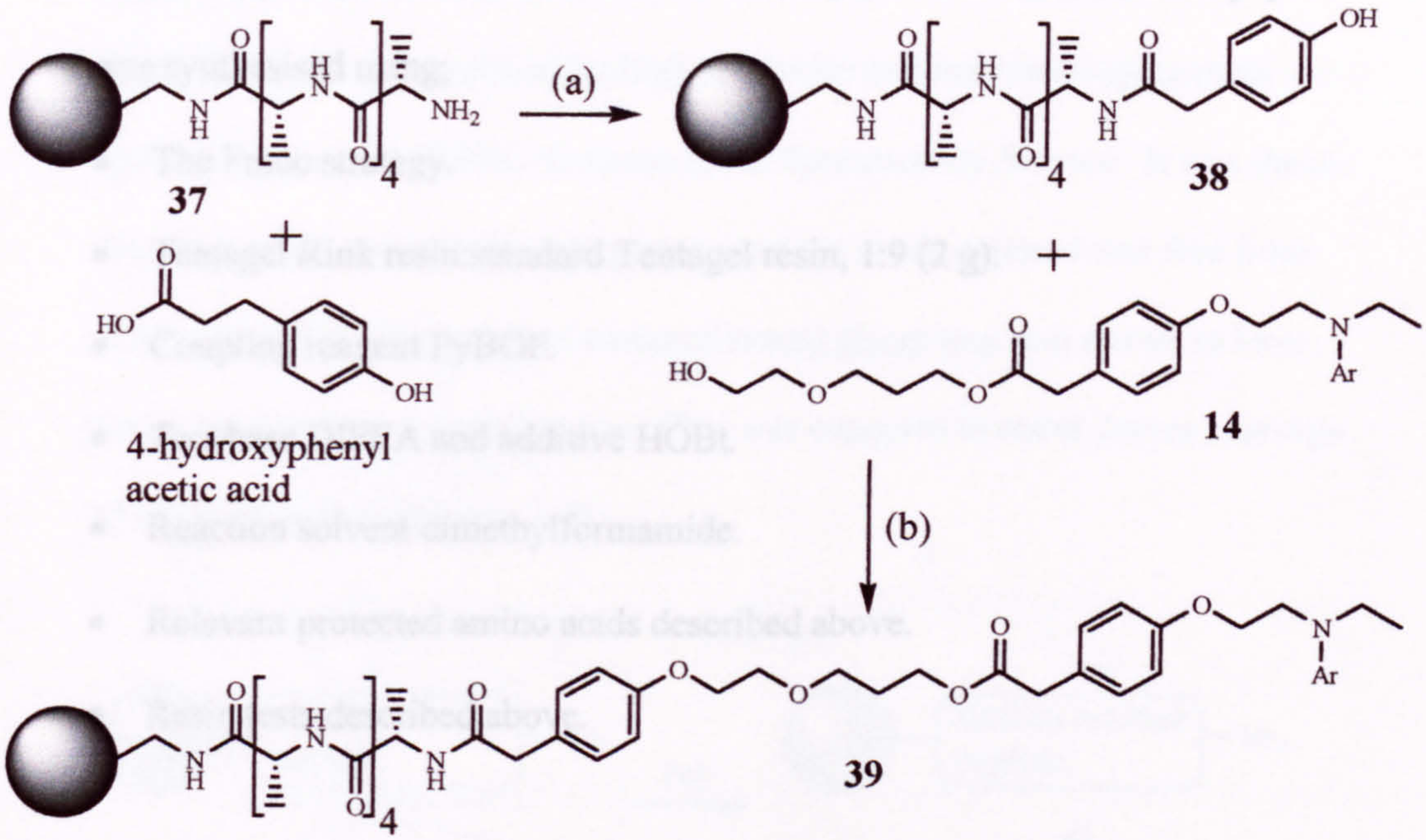
The pentapeptide was sequentially synthesised with the Fmoc strategy (Fmoc-L-Ala-OH) on Tentagel (1 g) using the coupling regent HBTU and the Kaiser resin test (Scheme 2.23).



(a) Tentagel (1.0 eq), α-N-alanine Fmoc (2.5 eq), PyBOP (2.5 eq), HOBt (2.5 eq), DIPEA (5.0 eq), dimethylformamide, room temperature, 2 hours, Kaiser test negative. (b) Piperidine:dimethylformamide, 1:19, room temperature, 20 minutes, Kaiser test positive.

Scheme 2.23. Manual synthesis of a pentapeptide on Tentagel *via* the Fmoc strategy.

Although each coupling stage took approximately 3 hours, the due to steric hindrance the last stage required double coupling. The Fmoc deprotection took approximately 20 minutes. After the supported pentapeptide **37** had been synthesised and its Fmoc protection removed to give a free terminal amino group, the pentapeptide was further functionalised with 4-hydroxyphenylacetic acid in the same manner Tentagel gel itself had been previously to give the supported pentapeptide derivative **38**. The dye-linker was then successfully coupled to this *via* Mitsunobu alkylation in the same manner previously undertaken for the functionalised Tentagel **35** to yield a dark red resin **39** when solvated in methanol, and a jet black resin when dry (Scheme 2.24).



(a) **37** (1.0 eq), 4-hydroxyphenylacetic acid (2.5 eq), PyBOP (2.5 eq), HOBt (2.5 eq), DIPEA (5.0 eq), dimethylformamide, room temperature, 2 hours, Kaiser test negative. (b) **38** (1.0 eq), DEAD (2.0 eq), PPh_3 (2.0 eq), **14** (2.5 eq), triethylamine (5.0 eq), tetrahydrofuran, room temperature, 1 hour.

Scheme 2.24. A high level of dye-linker loading was achieved giving a jet black resin.

The supported pentapeptide derivative **38** was then subjected to the coupling conditions with the DEAD omitted to reveal the resin was stained a light pink colour after light washing with methanol and dimethylformamide.

2.1.4.7 Synthesis of The Sixteen Residue Peptide

In view of this we were confident it would be possible to attach the dye-linker **14** to a derivative of the sixteen residue peptide which had been functionalised with

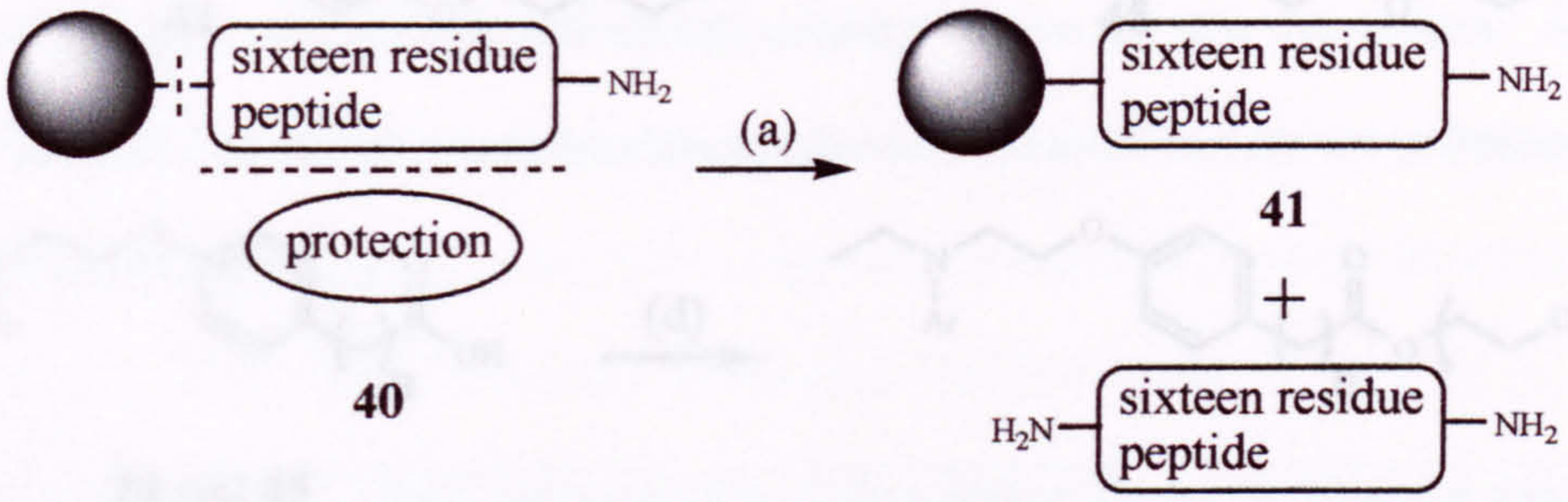
4-hydroxyphenylacetic acid *via* Mitsunobu alkylation. The sixteen unit peptide was synthesised using;

- The Fmoc strategy.
- Tentagel Rink resin:standard Tentagel resin, 1:9 (2 g).
- Coupling reagent PyBOP.
- *Tert*-base DIPEA and additive HOBt.
- Reaction solvent dimethylformamide.
- Relevant protected amino acids described above.
- Resin tests described above.

It was found that after residue four, the coupling times increased from 3 hours to 6 hours and after residue eight, from which point, double and triple coupling was required to achieve full conversion. This was due to the reactive terminal site of the peptide being sterically hindered as the peptide grew. After the last protected amino acid had been successfully coupled and its Fmoc protection removed, the bulk resin was thoroughly washed, dried under reduced pressure and stored under nitrogen to give a pale yellow resin **40** (Scheme 2.25). Note the peptides side chain protecting groups still remained.

A small sample (200 mg) was then subjected to a TIS:TFA, 1:99 solution. The reaction mixture was gently stirred using a magnetic stirrer bar for 2 hours after which time the resin supporting the fully deprotected peptide **41** was removed by filtration, thoroughly washed with dimethylformamide and methanol and dried under reduced pressure to give a pale yellow material. The filtrate was concentrated and then thoroughly dried under reduced pressure to give an off white powder **42** (5 mg) which was expected to contain the fully deprotected

peptide which had been cleaved from the resin upon cleavage of the acid labile Rink linker. **42** was analysed by high resolution tandem electrospray mass spectrometry at the EPSRC Swansea Mass Spectrometry Service. It was shown that the sixteen unit peptide was present and the crude material was free from shorter peptides. The terminal carboxylic acid group was also shown to have been converted to an amino group. This was expected to occur during cleavage of the Rink linker (Scheme 2.22).



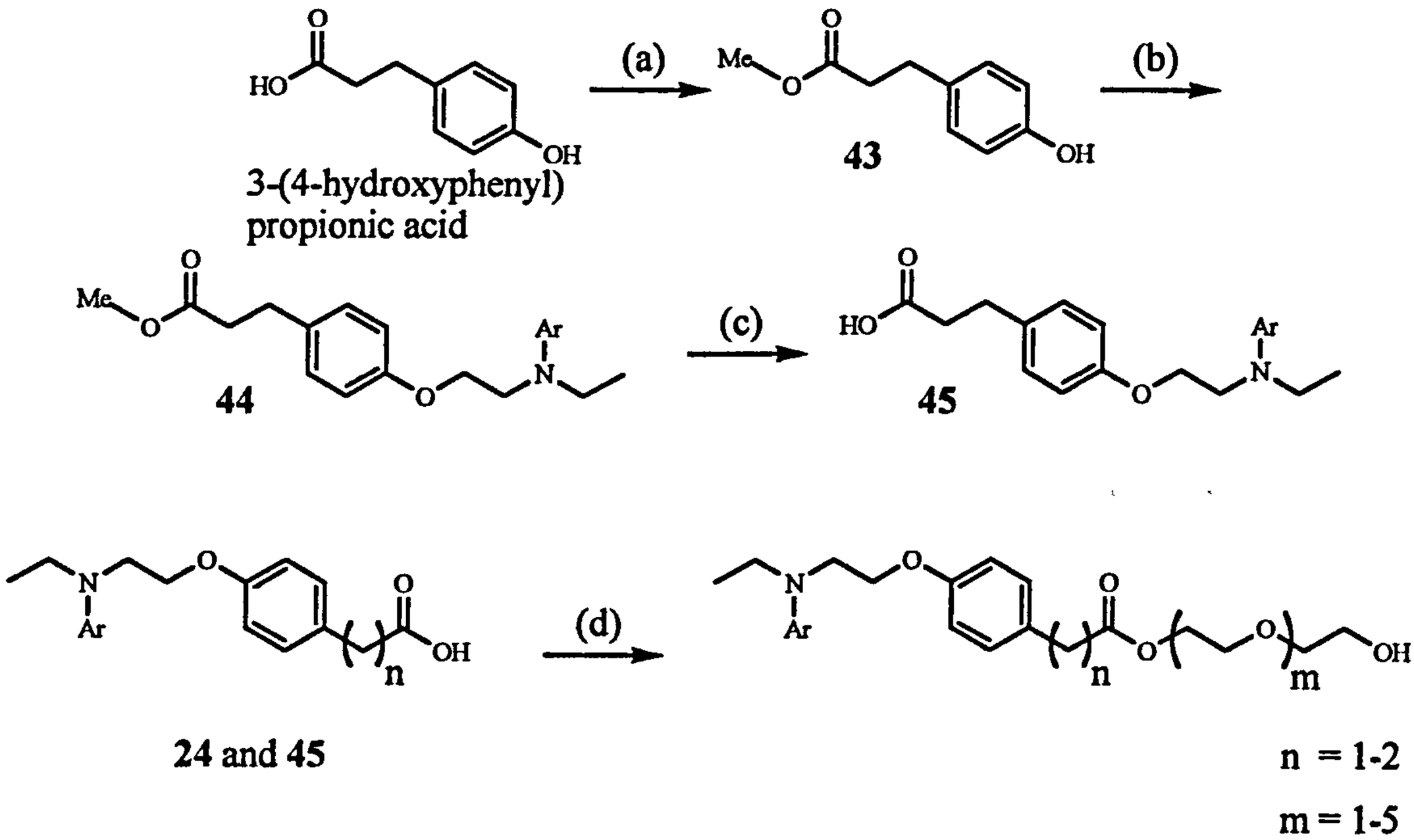
(a) TIS:TFA, 1:49, room temperature, 1 hour.

Scheme 2.25.. Removal of side chain protection and partial cleavage from the support.

2.1.5 Glycol Dye-Linkers

An important part of the intramolecular synthetic esterase model was to use a range of dye-linkers with varying lengths and we envisaged that modifying the acid derivative of disperse red **24** with di – hexaethylene glycol units would achieve this. A second acid derivative of disperse red **45** was also synthesised

and modified with the glycol units (Scheme 2.25). The substrates were synthesised whilst acquiring the mass spectrometry analysis of the peptide, since this took several weeks to complete.



(a) 3-(4-hydroxyphenyl) propionic acid (1.0 eq), sulphuric acid (catalytic), methanol, reflux, 3 hours, 94%. (b) **43** (1.0 eq), DEAD (1.0 eq), PPh_3 (1.0 eq), disperse red (0.9 eq), toluene, 80°C, 6 hours, 52%. (c) **44** (1.0 eq), lithium hydroxide (1.5 eq), tetrahydrofuran, room temperature, 12 hours, 40°C, 3 hours, 75%. (d) **24** (1.0 eq), DEAD (1.5 eq), PPh_3 (1.5 eq), glycol (1.5 eq), toluene, room temperature, 5 days, 68-81%: **45** (1.0 eq), DEAD (1.5 eq), PPh_3 (1.5 eq), glycol (1.5 eq), dichloromethane, 3 hours, 60-74%.

Scheme 2.26. Synthesis of the second acid derivative of disperse red and the ten glycol dye-linkers.

The second acid derivative of disperse red was initially synthesised from 3-(4-hydroxyphenyl) propionic acid. The carboxylic acid was protected by forming the methyl ester **43** by refluxing 3-(4-hydroxyphenyl) propionic acid, in methanol in the presence of acid for 3 hours. The methyl ester **43** was then

coupled to disperse red *via* Mitsunobu alkylation as before, except a longer reaction time of 6 hours was required (ca. 30 minutes) to give **44** in a moderate yield of 52%. The Mitsunobu alkylation reactions were never optimised and a major competing side product was formation of the corresponding dimer. Subsequent saponification with lithium hydroxide, gave the second acid derivative of disperse red **45** in a reasonable yield of 75%.

Upon accumulating reasonable amounts of both disperse red derivatives **24** and **45** the glycol units were individually coupled to give ten new dye-linkers. At this point we decided to abbreviate the dye-linkers to DLmn shown in Scheme 2.26 and Table 2.1.

Glycol unit, m	Ester component, n	Dye-linker, DLmn	Product number
2	1	DL_{21}	**46** (81%)
3	1	DL_{31}	**47** (71%)
4	1	DL_{41}	**48** (73%)
5	1	DL_{51}	**49** (78%)
6	1	DL_{61}	**50** (68%)
2	2	DL_{22}	**51** (74%)
3	2	DL_{32}	**52** (69%)
4	2	DL_{42}	**53** (70%)
5	2	DL_{52}	**54** (66%)
6	2	DL_{62}	**55** (60%)

Table 2.1. Nomenclature of the glycol dye-linkers.

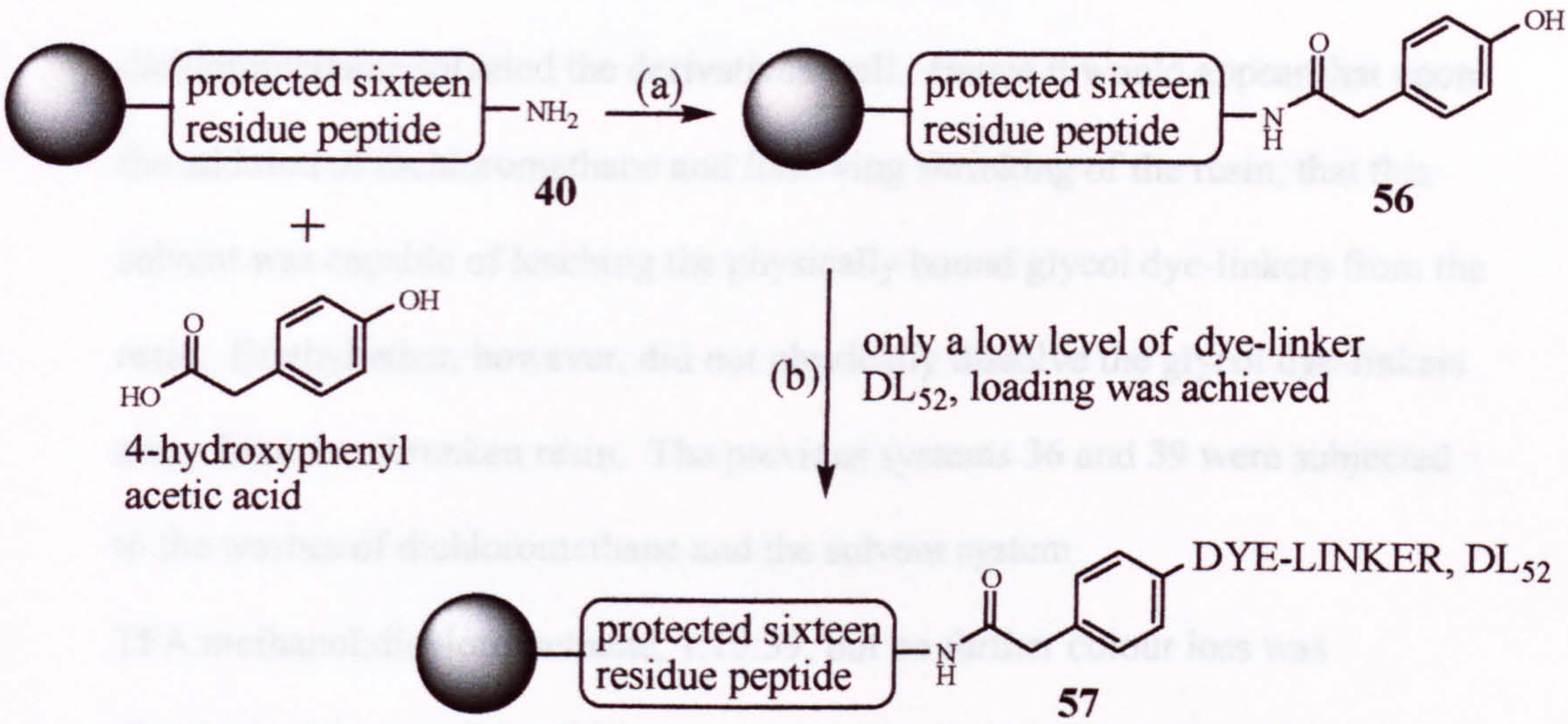
Note DL_{21} – DL_{61} were synthesised *via* Mitsunobu alkylation in toluene and the reactions took 5 days to achieve full conversion giving yields within the range of 68-81%. However, DL_{22} – DL_{62} were synthesised in dichloromethane and the reaction rate was dramatically increased; full conversion was achieved within 3 hours, giving yields within the range of 60-74%.

2.1.6 Attaching Dye-Linker DL_{52} to The Sixteen Residue Peptide

Having shown the peptide had been successfully synthesised we attempted to attach the glycol dye-linkers to the supported peptide (with side chain protecting groups) **40** *via* the methodology developed. The free amino group of the peptide was functionalised with 4-hydroxyphenylacetic acid to give **56** (Scheme 2.27).

The glycol dye-linker used in the initial studies was DL_{52} as a relatively large amount of material was available.

Upon subjecting **56** to the glycol dye-linker DL_{52} to the previous Mitsunobu conditions, a resin with a high level of loading affording jet black beads was obtained within 10 minutes and the colour remained throughout washes with dimethylformamide, methanol, toluene, diethyl ether and ethyl acetate. This was very encouraging although it was surprising a high level of loading was achieved within 10 minutes for a system so sterically hindered. Previous coupling with far less hindered systems had required a reaction time of approximately 2 hours.



(a) Supported peptide **40** (1.0 eq), 4-hydroxyphenylacetic acid (2.5 eq), PyBOP (2.5 eq), HOBt (2.5 eq), DIPEA (5.0 eq), dimethylformamide, room temperature, 2 hours, Kaiser test negative. (b) **56** (1.0 eq), DEAD (2.0 eq), PPh_3 (2.0 eq), **54** (2.5 eq), triethylamine (5.0 eq), tetrahydrofuran, room temperature, 1 hour.

Scheme 2.27. Attaching the glycol dye-linkers to the sixteen residue peptide.

However, when exposed to dichloromethane, it was suddenly discovered the colour began to rapidly leach away from the resin. The best solvent system to afford the colour loss was TFA:methanol:dichloromethane, 1:10:39. This was rather disappointing and it appeared that the glycol dye-linker was physically (rather than covalently) binding the peptide. It was clearly apparent the resin system was poorly solvated by diethyl ether and dichloromethane in which the resin colour would change from a deep red to jet black, the resin would appear to shrink in size and instead of being capable of moving freely in the solvent, would quickly rise to the surface and stick to edges of glassware. The difference between the two solvents (diethyl ether and dichloromethane) was that diethyl

ether poorly solvated the non-supported disperse red derivatives, whereas dichloromethane solvated the derivatives well. Hence it would appear that upon the addition of dichloromethane and following shrinking of the resin, that this solvent was capable of leaching the physically bound glycol dye-linkers from the resin. Diethyl ether, however, did not physically dissolve the glycol dye-linkers away from the shrunken resin. The previous systems **36** and **39** were subjected to the washes of dichloromethane and the solvent system TFA:methanol:dichloromethane, 1:10:39, but no further colour loss was observed. A range of conditions to optimise the chemical coupling of the glycol dye linker DL_{52} **54** to the supported derivative of the peptide **56** were investigated, the results from which are shown in Table 2.2.

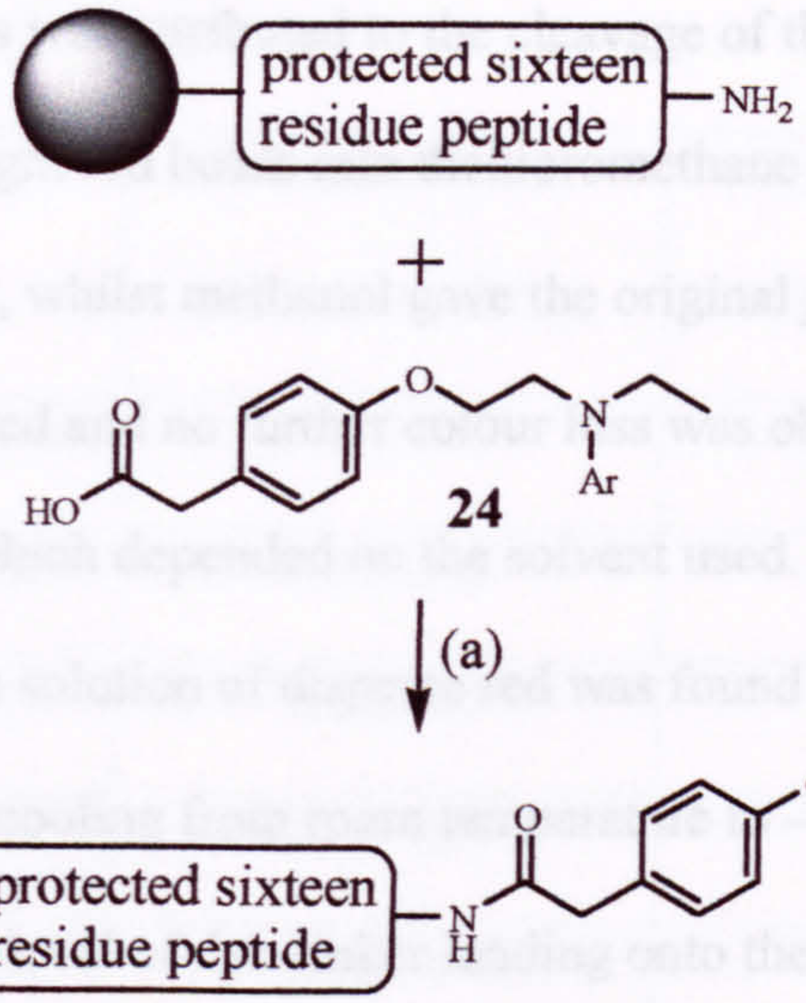
Solvent	Solvent Volume	Colour of Resin
Tetrahydrofuran	1.0 ml	Light Red
Tetrahydrofuran	0.5 ml	Bright red
Dichloromethane	1.0 ml	Light Pink
Dichloromethane	0.5 ml	Light Pink
Toluene	1.0 ml	Light Pink
Toluene	0.5 ml	Light Pink
Ethyl acetate	1.0 ml	Light Pink
Ethyl Acetate	0.5 ml	Light Pink

Table 2.2. Solvents and concentrations investigated in order to increase the level of loading.

Resin samples on a 10 mg scale were investigated where a large excess of the diethyl azodicarboxylate (DEAD), triphenylphosphine (PPh_3) and **54** were used and it was found the presence of triethylamine was crucial. The hindered base DIPEA was also investigated but a light pink resin was obtained in each case. Tetrahydrofuran was the solvent in which the highest level of coupling was obtained, and it was important to use a minimum of solvent. The best conditions yielded a resin that retained a moderate red coloration.

Although evidence indicated it was possible to chemically bind the glycol dye-linker DL_{52} to the supported peptide derivative, the level of loading was low and the coupling was difficult to reproduce. It was envisaged that the required coupling, might be afforded by formation of an amide bond between the peptide's terminal amino group and the carboxylate of an acid derivative of disperse red, by use of the highly efficient phosphonium coupling reagent PyBOP.

Therefore an attempt to couple the acid derivative **24** directly to the supported peptide **40** *via* the coupling reagent PyBOP, was undertaken (Scheme 2.28).



(a) Supported peptide **40** (1.0 eq), **24** (2.5 eq), PyBOP (2.5 eq), HOBt (2.5 eq), DIPEA (5.0 eq), dimethylformamide, room temperature, 2 hours.

Scheme 2.28. Direct coupling of **24** to the supported peptide *via* PyBOP.

Again a high level of loading appeared to be attained after 10 minutes and a jet black resin was obtained. However upon subjecting the resin to washes with various solvents including the solvent system TFA:methanol:dichloromethane, 1:10:39, the resin was found to remain a jet black. The TFA had been included in the solvent system to provide a proton source to help disrupt hydrogen bonding that may have been causing the dye-linkers to physically bind to the peptide. It was felt that this phenomenon would perhaps be even more relevant with regard to the acidic derivatives of disperse red. Note the acid derivative **24** was only sparingly soluble in dichloromethane. It was also important to examine how stable the supported peptide-disperse red system was to TFA because this was to be used in the final deprotected and partially cleaved peptide.

Remarkably, upon suspending the jet black resin **58** in TFA, the coloration changed to a brilliant bright red, although only slight colour was observed to

leach into the TFA (this was attributed to the cleavage of the Rink linker). Placing the brilliant bright red beads into dichloromethane changed the coloration to a dark red, whilst methanol gave the original jet black resin. This process could be repeated and no further colour loss was observed but a colour change was observed which depended on the solvent used. (Interestingly, during the Swern oxidation the solution of disperse red was found to lose a significant amount of colour upon cooling from room temperature to –78°C).

Thus it appeared a high level of dye-linker loading onto the supported peptide might now be quickly achieved with ease. Other solvents for undertaking the coupling were investigated (Table 2.3) and the physical binding of **24** to the supported peptide in various solvents were also investigated (Table 2.4). It was found that dimethylformamide was a suitable solvent and this was used for the future coupling of all the glycol dye-linkers. It was also found that **24** did physically stain the supported peptide in all solvents although this was easily removed in each case by washing with the TFA:methanol:dichloromethane, 1:10:39 solvent system.

This is an important point because during the deprotection of the supported peptide dye-linker systems with TFA, if hydrolysis occurred, **24** and **45** would be released. If the compounds **24** and **45** physically bound to the peptide throughout standard solvent washing, they would be present in future hydrolysis studies of the synthetic esterase systems. If they were then released during the studies, false results could be obtained.

Solvent Used In Coupling	Colour of Resin Before Washing	Colour of Resin After Washing*
Dimethylformamide	Jet Black	Jet Black
Tetrahydrofuran	Jet Black	Dark Red
Toluene	Jet Black	Dark Red
Ethyl Acetate	Jet Black	Dark Red
Dichloromethane	Jet Black	Dark Red

* Solvents used were methanol, dichloromethane, ethyl acetate and dimethylformamide.

Table 2.3. Coupling **24** to the supported peptide *via* PyBOP in various solvents.

Solvent Used	Colour of Resin Before Washing	Colour of Resin After Washing*	Colour of Resin After Washing**
Dimethylformamide	Jet Black	Light Red	Light Pink
Tetrahydrofuran	Jet Black	Light Red	Light Pink
Toluene	Jet Black	Dark Red	Light Pink
Ethyl Acetate	Jet Black	Dark Red	Light Pink
Dichloromethane	Jet Black	Light Red	Light Pink

* Solvents used were methanol, dichloromethane, ethyl acetate and dimethylformamide.

** The solvent system used was TFA:methanol:dichloromethane, 1:10:39.

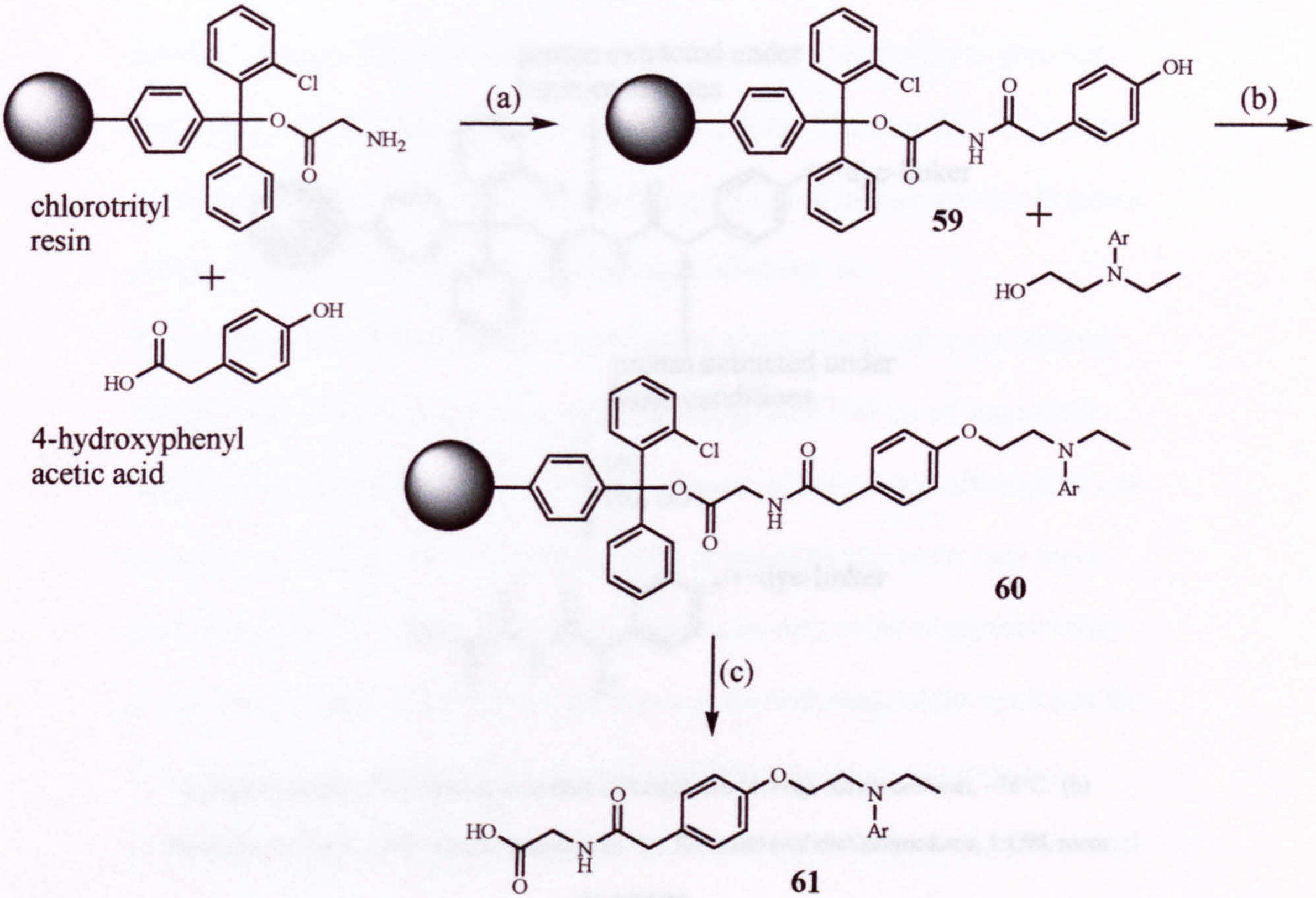
Table 2.4. Observing the physical staining of **24** to the supported peptide in various solvents.

2.1.7 Modifying The Dye-Linkers

2.1.7.1 Chlorotrityl Resin Method

It had been demonstrated that the glycol dye-linkers might be coupled to the supported peptide using the coupling reagent PyBOP. However, the glycol dye linkers would require their terminal primary hydroxy functionality to be modified to a free carboxylate. It had been shown previously that the oxidation of disperse red was troublesome and to modify all ten glycol dye-linkers would require a great deal of effort. Therefore a method using a commercially available polymer support preloaded with a glycine amino acid residue was investigated (Scheme 2.29).

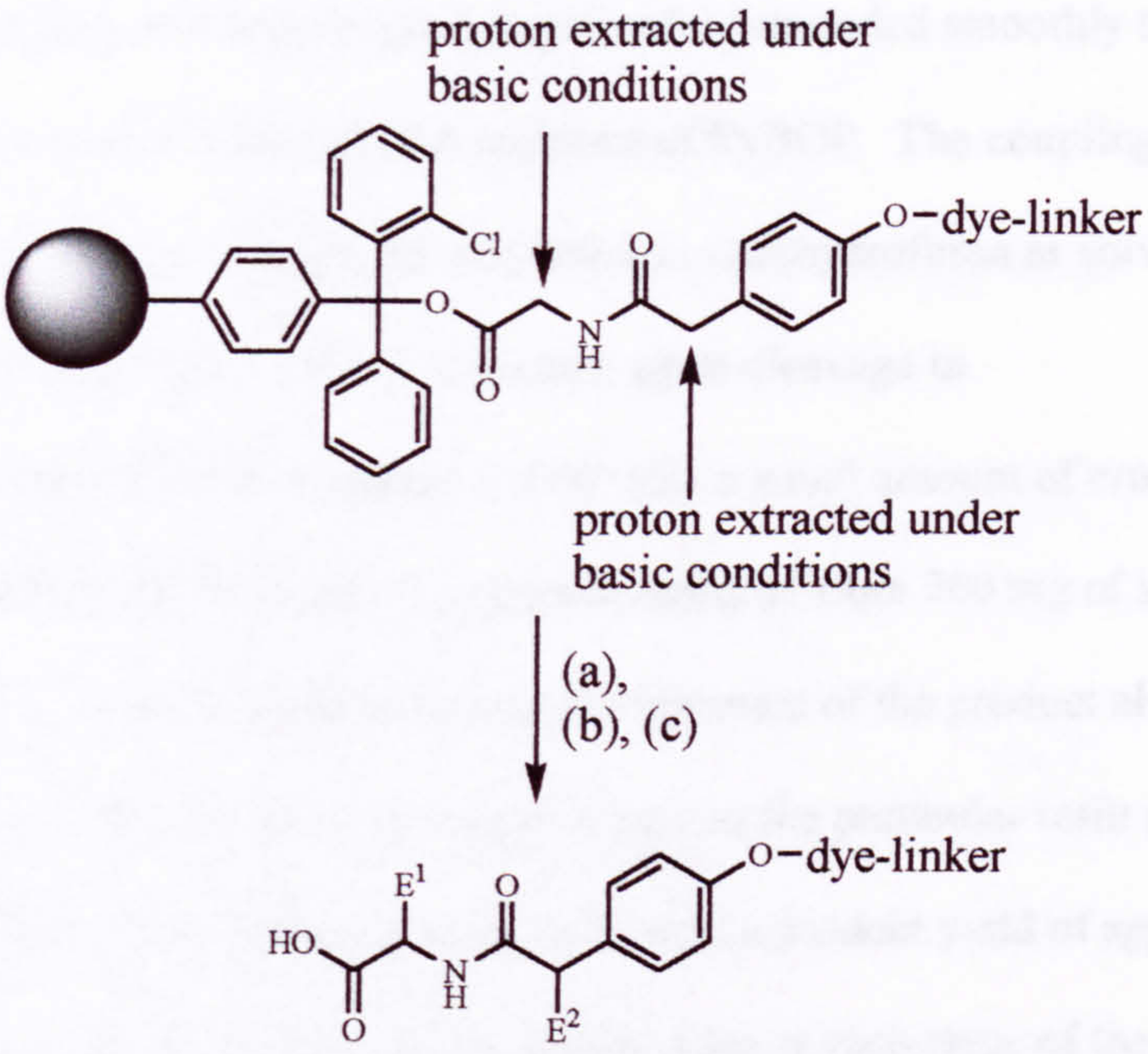
As described above, chlorotrityl resins[82,83] are highly acid labile. In this instance functionalising the free amino group of the supported glycine residue with 4-hydroxyphenylacetic acid to give **59** followed by additional coupling with the primary alcohol of disperse red gave a supported species **60**. When subjected to acidic conditions the chlorotrityl component was expected to cleave, yielding the new derivative **61**. The methodology would therefore quickly exchange the alcoholic function for a carboxy function through a short linker. It was envisaged that the system could also be used to create a combinatorial library of ester components for each individual glycol dye-linker (Scheme 2.30).



(a) Chlorotrityl resin (1.0 eq), 4-hydroxyphenylacetic acid (2.5 eq), PyBOP (2.5 eq), HOBt (2.5 eq), DIPEA (5.0 eq), dimethylformamide, room temperature, 2 hours, Kaiser test negative. (b) **59** (1.0 eq), DEAD (2.0 eq), PPh_3 (2.0 eq), disperse red (2.0 eq), triethylamine (5.0 eq), tetrahydrofuran, room temperature, 1 hour.

(c) TFA:methanol:dichloromethane, 1:1:98, room temperature, 5 minutes.

Scheme 2.29. Modifying the terminal functionality of the glycol dye-linkers *via* a chlorotrityl resin.



(a) Supported chlorotrityl dye-linker system (1.0 eq), LDA (1.0 eq), tetrahydrofuran, –78°C. (b) Electrophile (1.0 eq), -78°C to room temperature. (c) TFA:methanol:dichloromethane, 1:1:98, room temperature.

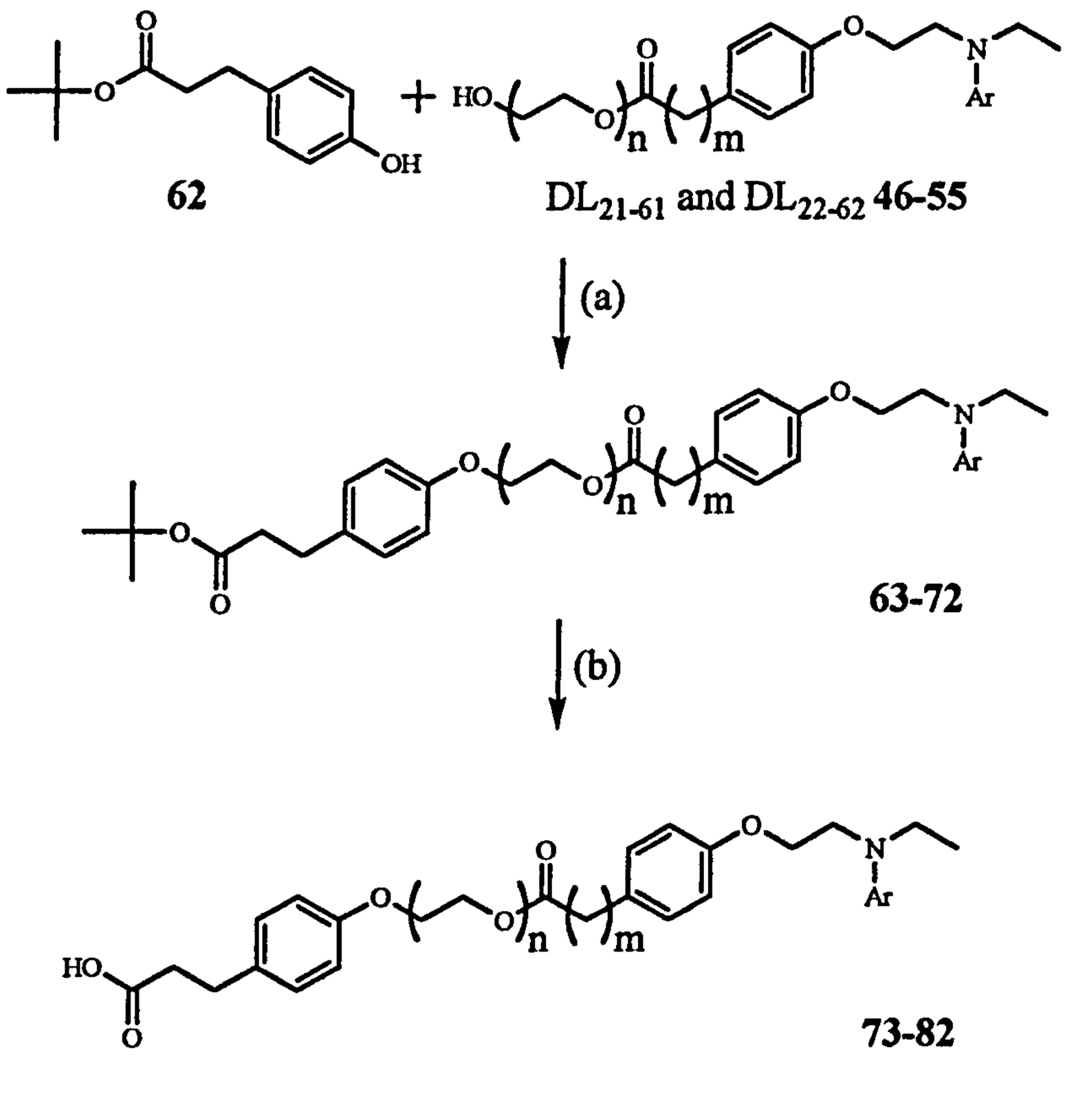
Scheme 2.30. The chlorotrityl resin could potentially provide a method to create a combinatorial library of glycol dye-linkers.

The chlorotrityl resin is known to stable to basic conditions. Deprotonation of the supported dye-linker with a suitable base such as lithium diisopropyl amide (LDA), splitting the bulk into several batches, quenching of each batch with a different electrophile, recombination of the batches and finally, cleavage from the resin could potentially yield a large range of glycol dye-linkers. However, two immediate problems with this method would be; a) controlling the site of deprotonation and b)controlling the mono C-alkylation.

During the synthesis of the system shown in Scheme 2.30, it was found that the initial coupling of 4-hydroxphenylacetic acid proceeded smoothly to give full conversion within 2 hours in the presence of PyBOP. The coupling of disperse red was effected by Mitsunobu alkylation in tetrahydrofuran as solvent, to give a red black resin within 1 hour. However, upon cleavage in TFA:methanol:dichloromethane, 1:1:98 only a small amount of crude material was obtained (approximately 7 mg crude material from 200 mg of supported substrate). Crude ^{1}H NMR indicated the presence of the product although it was not isolated. The low yield was surprisingly as the particular resin used had a fairly high level of loading (0.54 mmol/g) and a product yield of approximately 58 mg was expected if full conversion was seen at each stage of the synthesis for 200 mg of resin. It was felt the synthesis could be optimised, however, we decided to modify each glycol dye-linker manually and readdress the chlorotrityl resin method at a later date.

2.1.7.2 Manual Synthesis

The individual conversion of each glycol dye-linker was undertaken by using the *tert*-butyl ester of 3-(4-hydroxyphenyl) propionic acid **62** (Scheme 2.31) followed by deprotection with TFA. The product numbers of each derivative are summarised in Table 2.5.



(a) Dye-linker (1.0 eq), DEAD (1.5 eq), PPh_3 (1.5 eq), **62** (2.0 eq), dichloromethane, room temperature, 5 hours. (b) Dichloromethane:TFA, 1:99, room temperature, 5 minutes.

Scheme 2.31. Manual modification of each glycol dye-linker.

Dye-Linker	*Tert*- Butyl Derivative	Acid Derivative
DL_{21}	**63** (53%)	**73** (85%)
DL_{31}	**64** (55%)	**74** (87%)
DL_{41}	**65** (52%)	**75** (84%)
DL_{51}	**66** (53%)	**76** (79%)
DL_{61}	**67** (58%)	**77** (86%)
DL_{22}	**68** (57%)	**78** (77%)
DL_{32}	**69** (38%)	**79** (81%)
DL_{42}	**70** (47%)	**80** (84%)
DL_{52}	**71** (44%)	**81** (79%)
DL_{62}	**72** (56%)	**82** (82%)

Table 2.5. Nomenclature of the modified glycol dye-linkers.

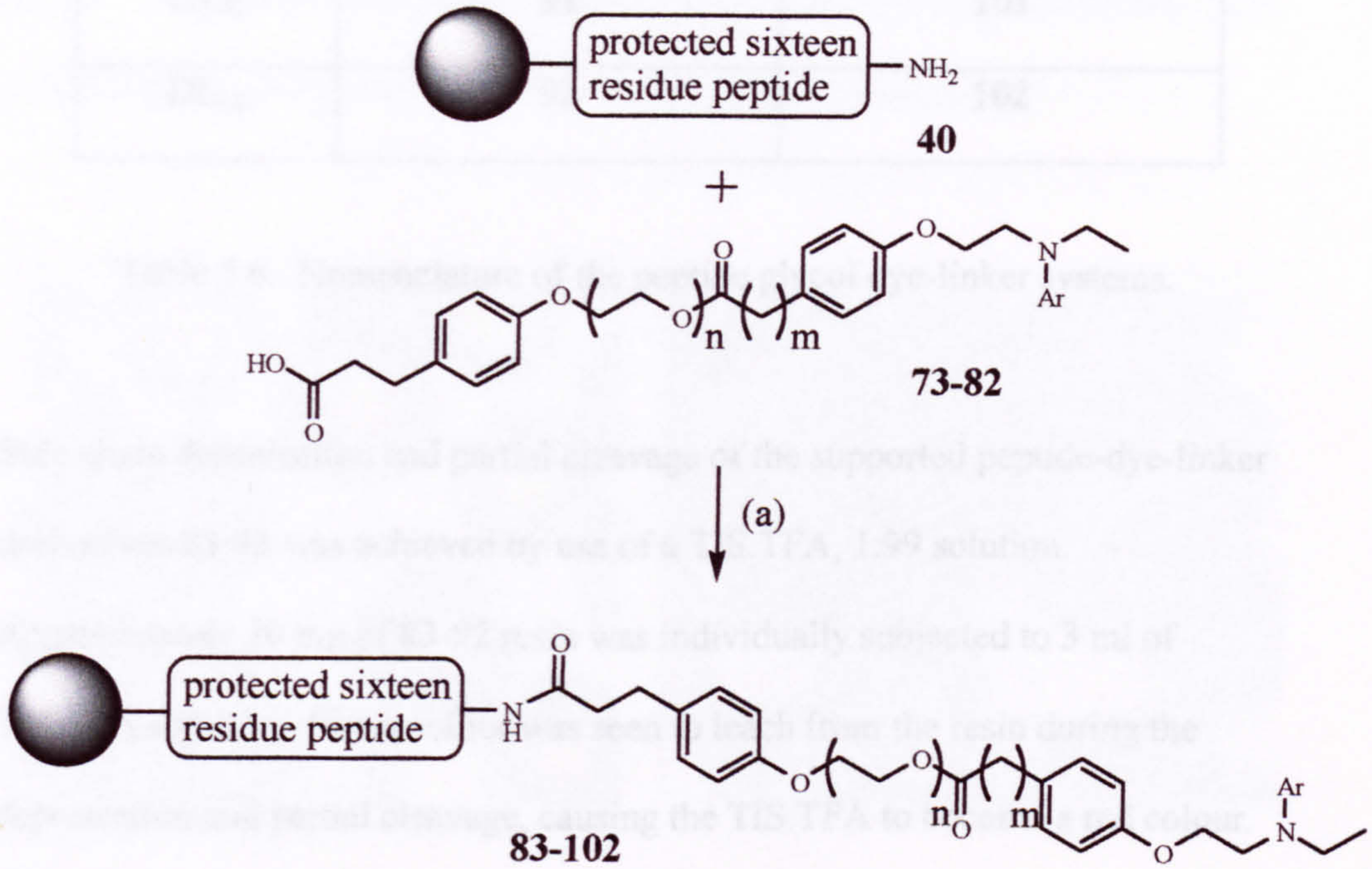
The *tert*-butyl ester was synthesised by a procedure reported by Ohta *et al.*[84] It involves forming the corresponding activated imidazole ester of 3-(4-hydroxyphenyl) propionic acid *via* reaction with carbonyl diimidazole in dimethylformamide at 40°C, and subsequent decarboxylation. The intermediate activated ester is then treated with 2.5 equivalents of *tert*-butanol and 2 equivalents 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) at 65°C for 48 hours to give the product **62** in 65% yield after isolation. This reaction was sometimes difficult to perform and yields would quite often be low for no apparent reason. For example, three reactions run in parallel, using the same reagents and in which all addition and reaction conditions were exactly the same, gave yields of

10%, 19% and 30% (these reactions were conducted at 40°C throughout for 12 hours using 1 equivalent of all reagents).

The coupling of **62** to the glycol dye-linkers **46-55** was undertaken *via* Mitsunobu alkylation using dichloromethane as solvent. The reactions took 5 hours to afford full conversion with yields between 38-58% (the major side products were the corresponding dimers). After isolation, the deprotection of the carboxyl group was cleanly achieved by dissolving the dye-linker derivative in a minimum of dichloromethane and adding the solution, to TFA containing 1% TIS. Full conversion could be observed by TLC (ethyl acetate:hexane, 4:1) within 5 minutes.

2.1.8 Attaching The Modified Dye-Linkers **73-82** to The Supported Peptide **40**

Attaching the modified dye-linkers **73-82** was accomplished using the same method described above for attaching **24** to the supported peptide (Scheme 2.28). The solvent of choice was dimethylformamide and the coupling reagent of choice was PyBOP (Scheme 2.32). It was found all the dye-linkers could be attached with a high level of loading within 3 hours and after washing with various organic solvents, a jet black resin was obtained to give the supported protected peptidyl systems **83-92** (Table 2.6).



(a) Supported peptide **40** (1.0 eq), dye-linker (2.5 eq), PyBOP (2.5 eq), HOBt (2.5 eq), DIPEA (5.0 eq), dimethylformamide, room temperature, 2 hours.

Scheme 2.32. Each glycol dye-linker **73-82** was directly coupled to the supported peptide *via* PyBOP. Peptidyl deprotection afforded **93-102**.

Dye-Linker	Protected Peptide On Support	Deprotected Peptide On Support
DL_{21}	**83**	**93**
DL_{31}	**84**	**94**
DL_{41}	**85**	**95**
DL_{51}	**86**	**96**
DL_{61}	**87**	**97**
DL_{22}	**88**	**98**
DL_{32}	**89**	**99**
DL_{42}	**90**	**100**

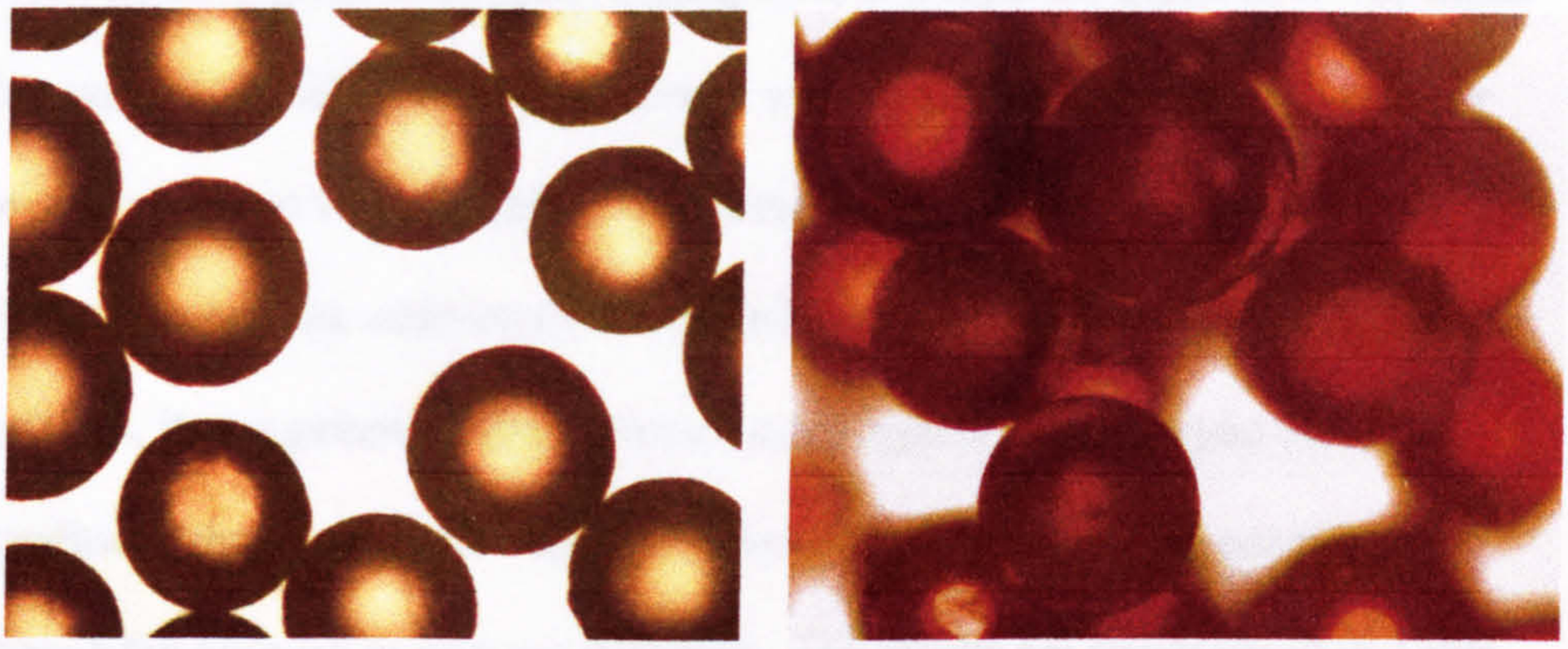
DL_{52}	**91**	**101**
DL_{62}	**92**	**102**

Table 2.6. Nomenclature of the peptide glycol dye-linker systems.

Side chain deprotection and partial cleavage of the supported peptide-dye-linker derivatives **83-92** was achieved by use of a TIS:TFA, 1:99 solution. Approximately 20 mg of **83-92** resin was individually subjected to 3 ml of TIS:TFA solution. Some colour was seen to leach from the resin during the deprotection and partial cleavage, causing the TIS:TFA to become a red colour. However, within the resin that was recovered, the majority of beads still had a high level of dye-linker loading and were a jet black colour. Some beads within the bead mixture had lost a significant amount of colour and become a light red. The beads that lost a significant amount of coloration were presumed to be the resin that had contained the Rink linker.

The red solution of TIS:TFA was concentrated and dried under reduced pressure to give in each case a red residue. All ten residues were analysed by low resolution mass spectrometry at the EPSRC Mass Spectrometry Service Swansea to confirm that each of the ten glycol dye-linker-peptide systems had been successfully synthesised and cleaved from the support. Picture 2.1.a shows the Tentagel resin prior to any loading and Picture 2.1.b shows the resin of **101**. The pictures were obtained by examining the resin under a microscope, to which a Cannon camera had been attached. The resin used had a high level of glycol dye-linker loading and normally appeared jet black. However, when examined under the microscope, a strong light source was used to illuminate the resin from

below. This had an effect which seemed to illuminate the individual beads from inside their structures and gave them a bright red appearance.



Picture 2.1. (a) Left: unfunctionalised Tentagel. (b) Right: Supported peptide dye-linker system **101**.

2.1.9 Analysis of The Ten Glycol Dye-Linker Peptide Systems

The methodology used to synthesise the intramolecular synthetic esterase systems had been accomplished and ten supported peptidyl systems with different linker lengths had been synthesised. A qualitative study was undertaken to investigate the degree of hydrolysis of these systems, in different solvents and pH values. The analysis involved swelling 50 small resin samples (0.5 mg) in small vials, in a buffer solution of pH 6 (0.5 ml), 50 resin samples in a buffer solution of pH 7 (0.5 ml) and 50 resin samples in a buffer solution of pH 8 (0.5 ml), at room temperature overnight. Within each batch of 50 resin samples, the 10 individual glycol dye-linker peptidic systems were represented 5

times. After being allowed to swell, the resin samples were then diluted with one of five organic co-solvents (1 ml); acetonitrile, tetrahydrofuran, dioxane, ethyl acetate and dimethylformamide. It was found that no hydrolysis was observed during the overnight swelling and, in a separate analysis, it was found that very little hydrolysis was observed when the supported glycol dye-linker peptidic systems were subjected to just the five organic solvents above.

However, upon the addition of the organic co-solvents to the swollen resin samples, it was generally found there was no hydrolysis but under certain conditions there was significant hydrolysis. The samples were not stirred or shaken but allowed to mix *via* diffusion. The results are summarised in Table 2.7.

	pH 6					
	MeCN	THF	Dioxane	MeOH	EtOAc	DMF
DL_{21}	x	x	x	x	x	x
DL_{31}	x	x	x	x	x	x
DL_{41}	x	x	x	x	x	x
DL_{51}	x	x	x	x	x	x
DL_{61}	x	x	x	x	x	x
DL_{22}	x	**S**	x	**S**	x	x
DL_{32}	x	x	x	x	x	x
DL_{42}	**S**	**S**	x	✓	x	✓
DL_{52}	✓	x	x	x	x	x
DL_{62}	x	x	x	x	x	x

	pH 7					
	MeCN	THF	Dioxane	MeOH	EtOAc	DMF
DL_{21}	x	x	x	x	x	x
DL_{31}	x	x	x	x	x	x
DL_{41}	x	x	x	x	x	x
DL_{51}	x	x	x	x	x	x
DL_{61}	x	x	x	x	x	x
DL_{22}	x	**S**	**S**	x	x	x
DL_{32}	x	x	x	x	x	x
DL_{42}	**S**	**S**	✓	**S**	x	x
DL_{52}	x	✓	**S**	**S**	x	x
DL_{62}	x	x	x	x	x	x

	pH 8					
	MeCN	THF	Dioxane	MeOH	EtOAc	DMF
DL_{21}	x	x	x	x	x	x
DL_{31}	x	x	x	x	x	x
DL_{41}	x	x	x	x	x	x
DL_{51}	x	x	x	x	x	x
DL_{61}	x	x	x	x	x	x
DL_{22}	x	x	x	x	x	x
DL_{32}	x	x	x	x	x	x
DL_{42}	✓	✓	S	x	x	x
DL_{52}	✓	✓	x	S	x	✓
DL_{62}	x	x	x	x	x	x

✓ **The most hydrolysis** ✓ **Reasonable hydrolysis**

S **Slight hydrolysis** x **Very slight or no hydrolysis**

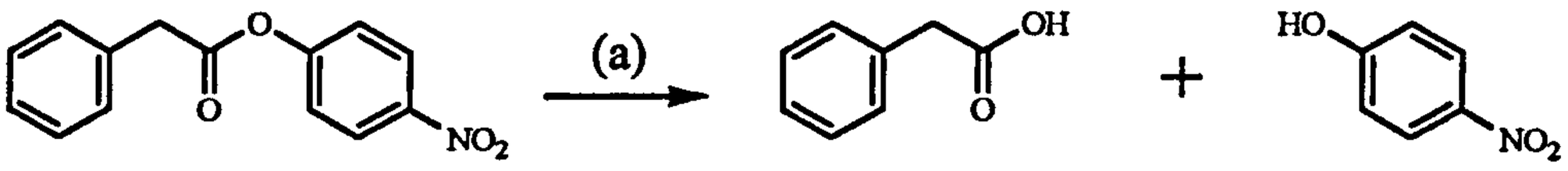
Table 2.7. Qualitative study to determine which conditions afforded the best intramolecular hydrolysis.

It was found that the peptidyl glycol dye-linker systems DL_{42} and DL_{52} gave the most hydrolysis whereas the systems DL_{21} to DL_{61} gave no or very slight hydrolysis. The system DL_{42} when subjected to the organic co-solvent, dioxane at pH 7, was found to give the most hydrolysis. The second and third most active systems were DL_{52} in tetrahydrofuran at pH 8 and acetonitrile at pH 6 respectively although the difference between the three was very slight. The analysis of hydrolysis was undertaken by visually comparing the intensity of the solutions red colour and an accurate qualitative analysis was not conducted. It is also important to note that none of the systems gave total hydrolysis in which colourless resin would have been obtained. The amount of colour loss was purely comparative and a significant level of loading remained throughout, leaving resin a dark red colour. The solution of the systems which had yielded

the most colour loss were examined by TLC (ethyl acetate:hexane, 4:1) and the only compounds present, were the corresponding hydrolysis products, **24** and **45** disperse red derivatives. It was noticed that most of the hydrolysis occurred within the first two hours and very slight increases were observed over a further 3 days.

2.1.10 Intermolecular Investigation

Although complete hydrolysis to colourless beads had not been achieved, it was encouraging that some systems were more active than others. This indicated the length of dye-linker and the ester being cleaved by intramolecular hydrolysis, was important. A further qualitative examination undertaken involved the intermolecular hydrolysis of the *p*-nitrophenyl ester **103** by the supported deprotected peptide **41** (Scheme 2.33).



phenylacetic acid 4-nitrophenyl ester **103**

phenylacetic acid

4-nitrophenol

Scheme 2.33. Hydrolysis of the ester gave phenyl acetic acid and 4-nitrophenol. Both products are UV active and can be detected by TLC.

The two organic solvents, acetonitrile and dioxane, had been shown in the previous studies to be suitable co-solvents to effect intramolecular hydrolysis.

These were used in the intermolecular analysis. Small resin samples of the supported peptide (0.5 mg, ~ 3.0 x 10^{-3} mmol) were again swollen overnight in buffer solutions (1 ml) of pH 6, 7 and 8. After swelling, **103** (0.5 mg, 1.9 mmol) dissolved in the appropriate solvent (0.5 ml) was added. Identical blank experiments were also run in parallel, where the presence of the peptide had been omitted. The hydrolysis of the *p*-nitrophenyl ester was followed by observing the formation of *p*-nitrophenol by TLC (ethyl acetate:hexane, 2:3) and the results are summarised in Table 2.8.

	pH 6	
	No Peptide Present	Peptide Present
Dioxane	S	S
Acetonitrile	x	✓
	pH 7	
	No Peptide Present	Peptide Present
Dioxane	✓	✓
Acetonitrile	✓	✓
	pH 8	
	No Peptide Present	Peptide Present
Dioxane	S	S
Acetonitrile	S	S

✓ **Complete hydrolysis**

S **Slight or incomplete hydrolysis**

x **no hydrolysis**

Table 2.8. Intermolecular hydrolysis of **103** in the presence of the supported peptide.

It was found that for each condition except one, that if hydrolysis was observed in the presence of the peptide, the same level of hydrolysis was observed without the peptides presence. The exception was when the hydrolysis was undertaken at

pH 6 using acetonitrile as co-solvent. No hydrolysis was observed without the peptide present, however complete hydrolysis was observed when the peptide was present. Regrettably the rate of hydrolysis in each case was slow and the experiments were observed over a ten day period.

Having established one particular set of conditions under which hydrolysis of **103** was only observed in the presence of the peptide, the experiment was repeated using a larger ratio of supported peptide (10 mg, ~ 60 x 10^{-3}). It was found on closer examination that very slight hydrolysis of the *p*-nitrophenyl ester occurred within the first 24 hours. The intensity ratio of the UV spots on TLC corresponding to **103** and to *p*-nitrophenol, as approximately 20:1 respectively. Over a further 4 days the ratio increased to approximately 1:1 and a strong conversion had been observed by day 6 giving a ratio of 1:4. The rate of hydrolysis of the remaining *p*-nitrophenyl ester then appeared to slow down again and a further 4 days were required to give complete conversion. A blank experiment (omitting the peptide) was again run in parallel and the presence of *p*-nitrophenol was not observed.

This was interesting as it appeared that the peptide required a period of time to become activated upon which the rate of hydrolysis increased until approximately 80% of the substrate had been converted to its hydrolysis products. The rate then appeared to slow down again during the final hydrolysis of remaining substrate. Complete hydrolysis had again taken 10 days clearly indicating that if the peptide was catalysing the hydrolysis, it was not very efficient.

One explanation for the apparent increase and decrease of the peptide's activity could be that initially the peptide required 24 hours to form an active secondary structure after which it assisted the hydrolysis of **103**. Subsequently a relative increase in activity was observed where the peptide was attacking **103** in a nucleophilic manner releasing the *p*-nitrophenol. However, the peptide then suffers product inhibition, possibly due to its active site being acylated by the phenylacetic acid in a stable manner and subsequent hydrolysis of the remaining substrate relied upon the rate at which the peptide could be regenerated. Note, at no time was the presence of phenylacetic acid observed during the hydrolysis although an unidentified baseline product did accompany cases of successful hydrolysis.

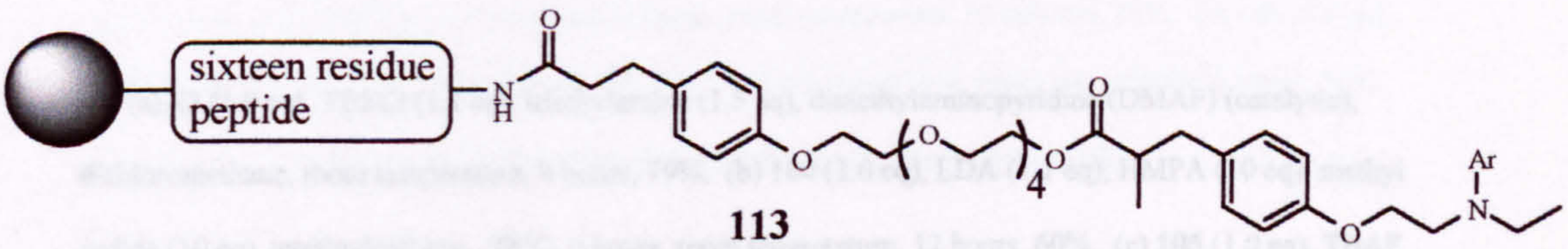
If the peptide was indeed successfully aiding the hydrolysis of **103** *via* a single active component, it could possibly have been operating in a catalytic sense because an excess of substrate was being processed. Further work is however, necessary to prove the peptide retained its original composition and additional investigations would certainly be necessary to prove whether an increase of reaction rate was being observed. The active components of the peptide's structure would also need to elucidated. It is known the imidazole group of histidine is capable of catalysing the hydrolysis of similar esters.[85,86] The hydrolysis we observed may have been due to the histidine residue within the peptide. The reduced rate of hydrolysis could then, for example, be rationalised to the binding of the hydrolysis products to the peptide and causing steric hindrance to the imidazole group of the histidine.

However, our concern during this study had been to demonstrate that the supported peptide was capable of inducing hydrolysis of an ester in order to

support the previous intramolecular studies. It was rationalised that further detailed studies of intermolecular hydrolysis should be undertaken, possibly with a far more active peptide sequence, if one should be realised.

2.1.11 Synthesis of a Chiral Ester Peptidic System

Having found that the optimal glycol dye-linker length to be DL_{52} and favourable hydrolysis conditions to effect its intramolecular hydrolysis, we envisaged it would be prudent to investigate if it was possible to observe if enantioselective control was being expressed during the hydrolysis. This was undertaken by synthesising another supported peptidic DL_{52} system where a chiral center was incorporated alpha to the carbonyl on the ester being hydrolysed (Figure 2.5). A racemic center was created and it was envisaged that any enantioselective control during the hydrolysis would be evaluated *via* chiral HPLC analysis.

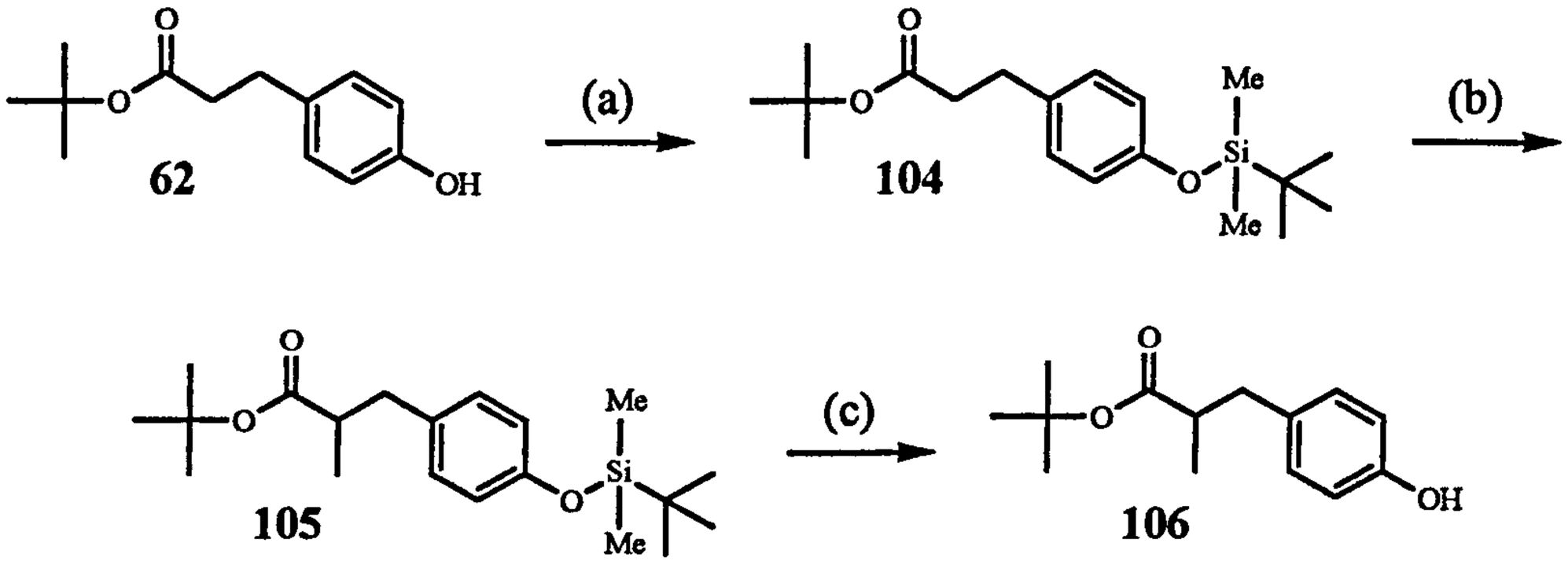


113

Figure 2.5. Supported peptidic DL_{52} system involving a chiral ester.

The synthesis of the racemic chiral glycol dye-linker **111** was initially undertaken by protecting the hydroxyphenyl group of **62** with *tert*-butyl

dimethylsilyl chloride in a reasonable yield of 79% (Scheme 2.34). Formation of the enolate of **104** *via* deprotonation alpha to the carbonyl with lithium diisopropyl amide at –78°C, followed by quenching at –78°C with a ten fold excess of methyl iodide in the presence of a ten fold excess of hexamethylphosphotriamide (HMPA) afforded **105** in a 60% yield. The HMPA was required to reduce unwanted side products arriving from polyalkylation of the enolate intermediate, and di-carbon and oxygen alkylation during the methyl iodide quenching.[87] The silyl protecting group of **105** was then removed with tetrabutylammonium fluoride in tetrahydrofuran. The deprotection was very quick and full conversion was observed within 3 minutes to give **106** with a yield of 82%.

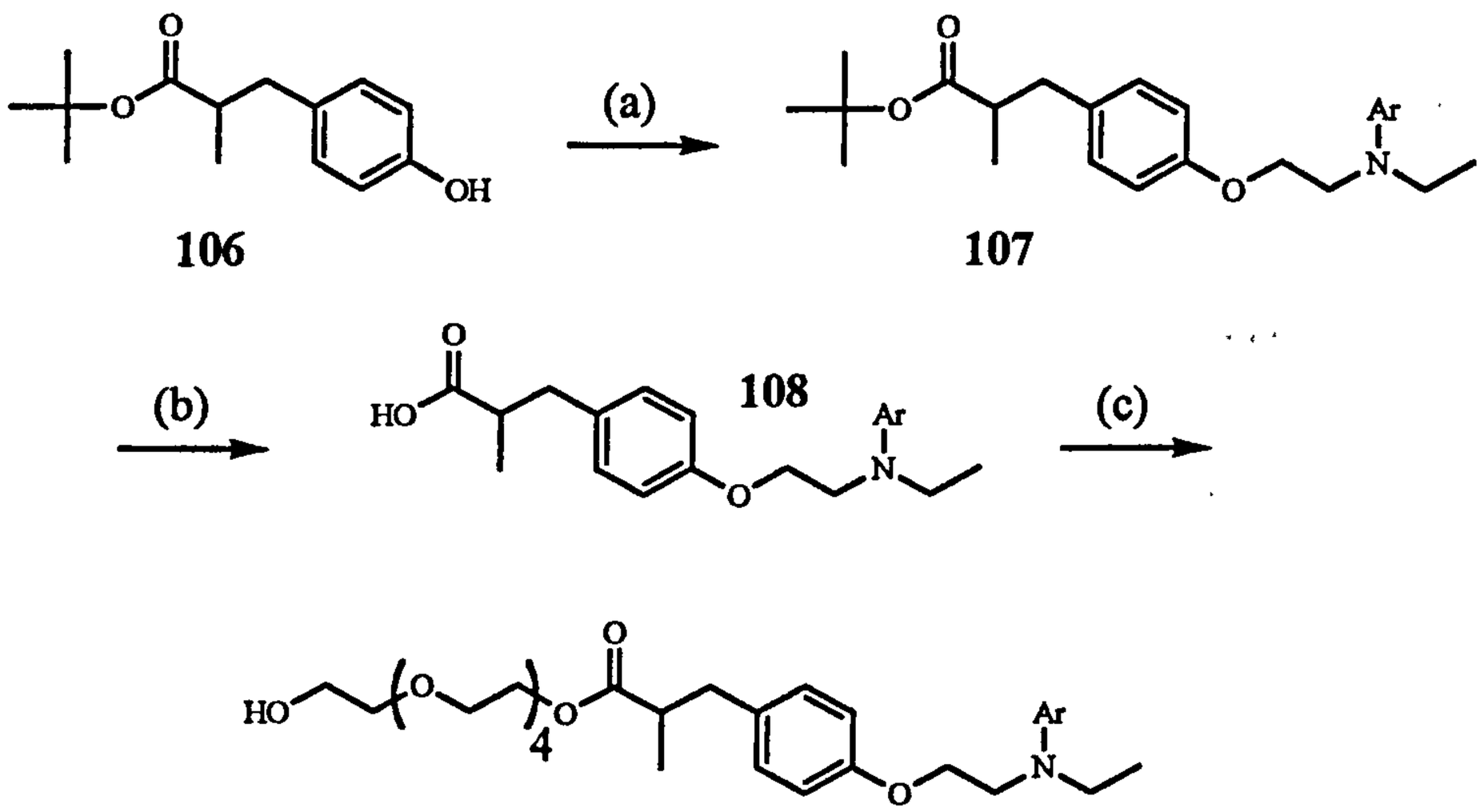


(a) **62** (1.0 eq), TBSCl (1.1 eq), triethylamine (1.5 eq), dimethylaminopyridine (DMAP) (catalytic), dichloromethane, room temperature, 8 hours, 79%. (b) **104** (1.0 eq), LDA (1.1 eq), HMPA (10 eq), methyl iodide (10 eq), tetrahydrofuran, -78°C, 6 hours, room temperature, 12 hours, 60%. (c) **105** (1.0 eq), TBAF (1.0 eq), tetrahydrofuran, room temperature, 20 minutes, 82%.

Scheme 2.34. Introducing a racemic chiral center α- to the carbonyl of **62**.

The coupling of **106** to disperse red was successfully achieved *via* Mitsunobu alkylation using dichloromethane as solvent (Scheme 2.35). Full conversion was

observed within 3 hours to **107** with a yield of 33%. It was found the major side product was the dimer of disperse red. Removal of *tert*- butyl protection to afford **108** was successfully undertaken with a reasonable yield of 81% by dissolving the substrate in a minimum of dichloromethane and direct delivery to a solution of TIS:TFA, 1:49. It was found if a higher concentration of TIS was present or too much dichloromethane was used to dissolve the substrate, efficient deprotection was difficult to achieve.

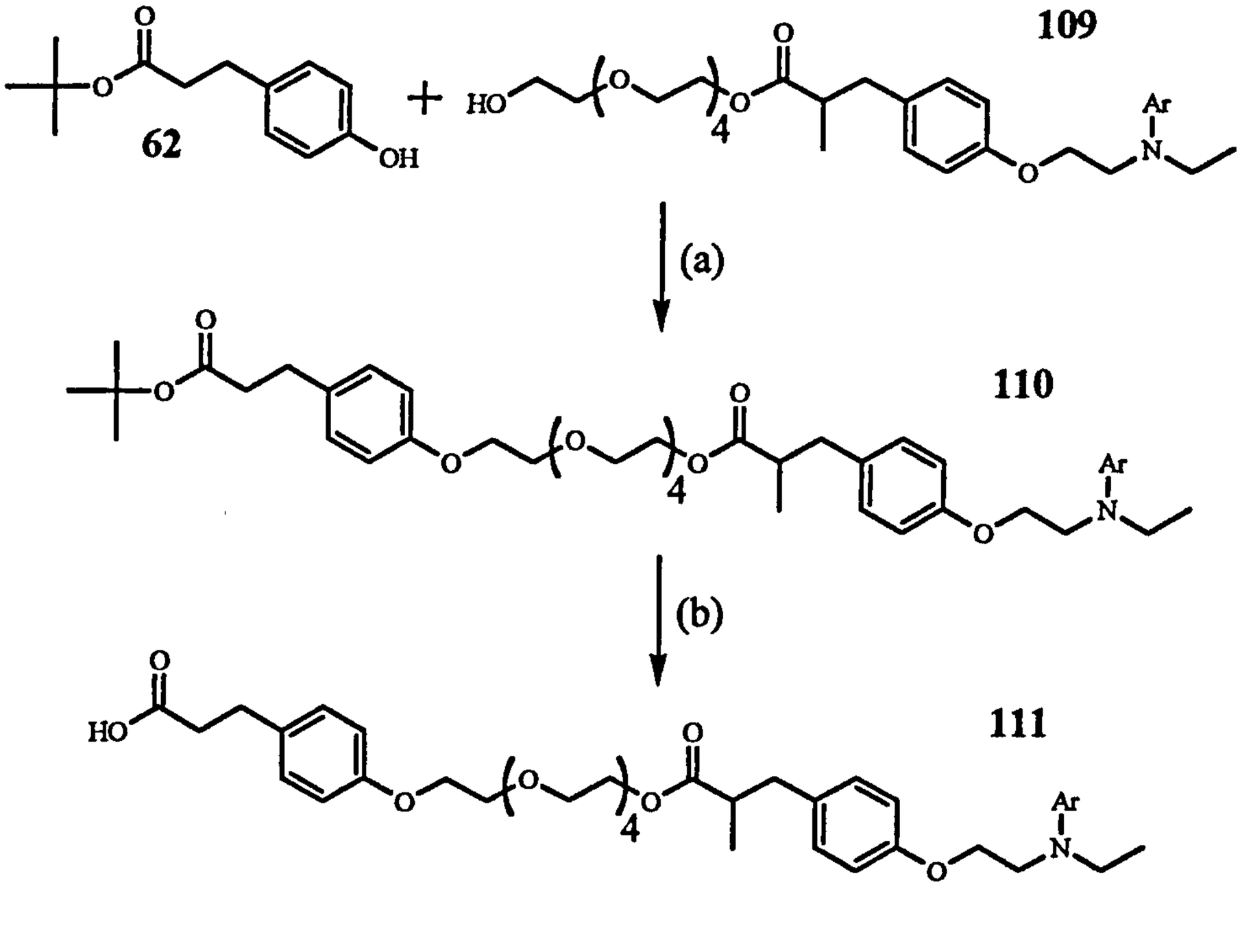


(a) **106** (1.0 eq), DEAD (1.0 eq), PPh_3 (1.0 eq), disperse red (1.0 eq), dichloromethane, room temperature, 3 hours, 33%. (b) TIS:TFA, 1:49, dichloromethane, room temperature, 10 minutes, 81%. (c) **108** (1.0 eq), DEAD (1.1 eq), PPh_3 (1.1 eq), pentaglycol (1.3 eq), dichloromethane, room temperature, 4 hours, 56%.

Scheme 2.35. Synthesis of the racemic dye-linker.

The coupling of **62** to **109** was also afforded *via* Mitsunobu alkylation with a moderate yield of 59% using dichloromethane as the solvent and the final deprotection was again achieved by dissolving the substrate in a minimum of

dichloromethane followed by addition to a solution of TIS:TFA, 1:49. The removal of the *tert*-butyl group gave **111** in a 79% yield (Scheme 2.36).

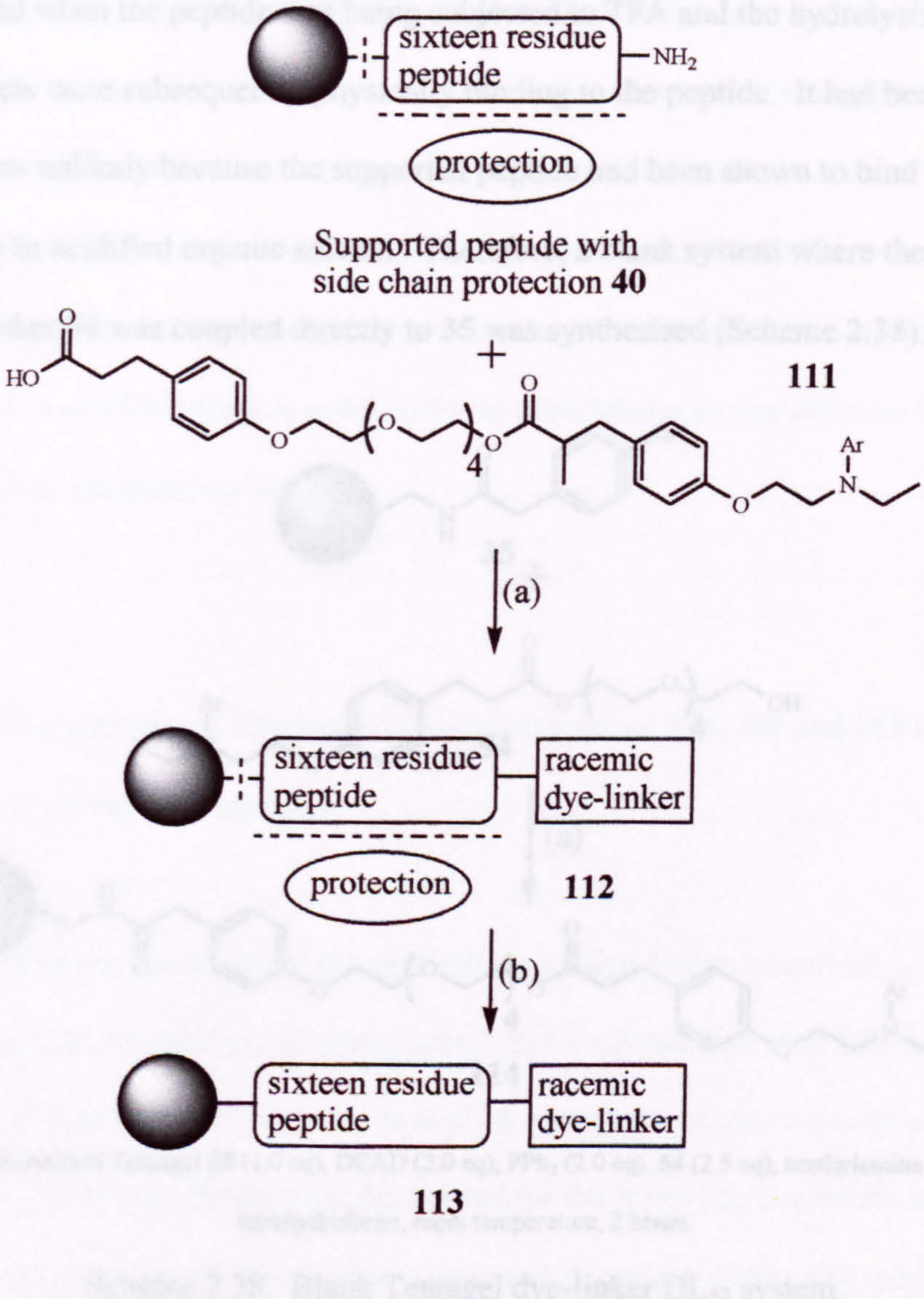


(a) **63** (1.0 eq), DEAD (1.1 eq), PPh_3 (1.1 eq), **110** (1.0 eq), dichloromethane, room temperature, 2 hours, 59%. (b) TIS:TFA, 1:49, dichloromethane, room temperature, 10 minutes, 79%.

Scheme 2.36. Deprotection of the final racemic glycol dye-linker.

Having synthesised the chiral glycol dye-linker **111**, it was coupled to the supported side chain protected peptide **40** (10 mg,) using the coupling reagent PyBOP in the same manner as above. The solvent of choice was dimethylformamide and an excellent level of loading was achieved after gentle stirring at room temperature for 2 hours to give a jet black resin **112**. Side chain protection was afforded by gently stirring **112** (9 mg) at room temperature for 1 hour in a solution of TIS:TFA, 1:49. After subjecting the resin to washes with

various solvents including the solvent system TFA:methanol:dichloromethane, 1:10:39, the resin was found to retain a high level of loading, giving the supported deprotected peptidic system **113** as a jet black resin (Scheme 2.37).

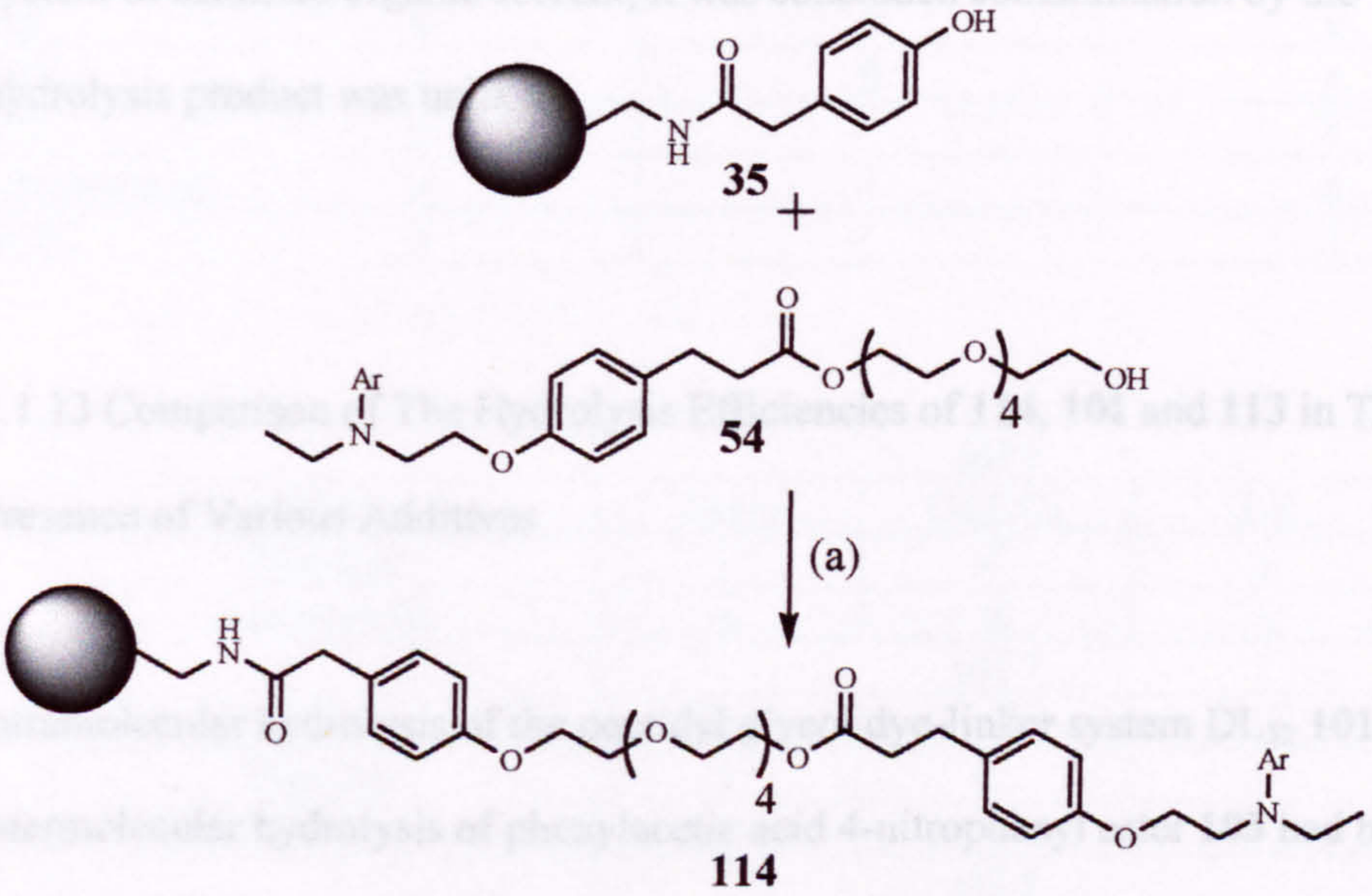


(a) Supported peptide (1.0 eq), dye-linker (2.5 eq), PyBOP (2.5 eq), HOBt (2.5 eq), DIPEA (5.0 eq), dimethylformamide, room temperature, 2 hours. (b) TIS:TFA, 1:49, room temperature, 1 hour.

Scheme 2.37. Deprotection of the supported peptidyl racemic glycol dye-linker.

2.1.12 Synthesis of a Blank Dye-Linker DL_{52} System

We were concerned during the of the hydrolysis studies that false results might have been observed if the esters in the peptidyl dye-linker systems were being cleaved when the peptide was being subjected to TFA and the hydrolysis products were subsequently physically binding to the peptide. It had been shown this was unlikely because the supported peptide had been shown to bind **24** poorly in acidified organic solvent. Therefore, a blank system where the glycol dye-linker **54** was coupled directly to **35** was synthesised (Scheme 2.38).



(a) Functionalised Tentagel **35** (1.0 eq), DEAD (2.0 eq), PPh_3 (2.0 eq), **54** (2.5 eq), triethylamine (5.0 eq), tetrahydrofuran, room temperature, 2 hours.

Scheme 2.38. Blank Tentagel dye-linker DL_{52} system.

A similar system **36** had already been synthesised with the dye-linker **14** and this could have been used in the investigations. However, it was envisaged that it would be prudent to use the same dye-linker currently under investigation. The

blank Tentagel dye-linker DL_{52}, system **114** was synthesised by standard Mitsunobu alkylation conditions above. After 2 hours a jet black resin was acquired which retained its loading upon several washes with various organic solvents and the solvent system TFA:methanol:dichloromethane, 1:10:39. **114** was then subjected to the TIS:TFA solution used to deprotect the peptidyl systems and it was found that no colour loss was observed over a period of 4 hours which was four times longer than the peptidyl deprotection stage. A very slight light pink solution was acquired after 24 hours in solution.

In view of this and that the hydrolysis product **24** did not bind the peptidyl system in acidified organic solvent, it was concluded contamination by the hydrolysis product was unlikely.

2.1.13 Comparison of The Hydrolysis Efficiencies of **114**, **101** and **113** in The Presence of Various Additives

Intramolecular hydrolysis of the peptidyl glycol dye-linker system DL_{52} **101** and intermolecular hydrolysis of phenylacetic acid 4-nitrophenyl ester **103** had both occurred with relatively high levels at pH 6 when acetonitrile was used as a co-solvent. These conditions were again used for the following qualitative analysis.

Small resin samples (0.5 mg) of the supported glycol dye-linker **114**, peptidyl glycol dye-linker **101** and the peptidyl glycol racemic dye-linker **113** were individually swollen in a buffer solution (0.5 ml) of pH 6 overnight. Various additives were then introduced (~ 2 μmol) followed by acetonitrile (0.5 ml). The results of the subsequent hydrolysis are summarised in table 2.9.

	Acetonitrile at pH 6		
Additive	Support-DL_{52} **114**	Support-peptide-DL_{52} **101**	Support-peptide-chiral DL_{52} **113**
1 $Ce_2(CO_3)_3$	x	✓	x
2 Cs_2CO_3	x	✓	**S**
3 CuCN	x	✓	x
4 $Pb(NO_3)_2$	x	x	x
5 NaF	x	✓	x
6 $AlCl_3$	x	x	x
7 LiI	x	*✓	x
8 $ZnCl_2$	x	✓	x
9 $CuBr_2$	x	✓	x
10 NaOAc	x	✓	x
11 $AuCl_3$	x	x	x
12 $CeCl_3$	x	x	x
13 $FeCl_3$	x	x	x
14 CuI	x	x	x
15 $CaCl_2$	x	**S**	x
16 KCl	x	✓	x
17 Vancomycin	x	✓	x
18 $ScTf_3$	x	✓	x

	pH 6		
Additive	None	Cs_2CO_3	LiI
Dioxane	x	✓	x
Acetonitrile	x	**S**	x
	pH 7		
Additive	None	Cs_2CO_3	LiI
Dioxane	x	✓	x
Acetonitrile	x	**S**	x
	pH 8		
Additive	None	Cs_2CO_3	LiI
Dioxane	x	✓	x
Acetonitrile	x	✓	x

✓ The most hydrolysis **✓ Reasonable hydrolysis**
S Slight hydrolysis **x Very slight or no hydrolysis**
*** strongest yet. The others were comparable to the previous systems.**

Tables 2.9 and 2.10. Qualitative Study to Determine if simple additives could enhance hydrolysis.

Several points of interest were evident:

- No hydrolysis occurred in any of the conditions for the supported glycol dye-linker system **114**. This was encouraging because it confirmed the presence of the peptide was important.
- For the majority of the conditions investigated, the same level of hydrolysis that had been experienced in the previous intramolecular studies, was also observed. However, for one example (additive lithium iodide) the highest level of hydrolysis given by any system, was observed. Several additives prevented any hydrolysis from occurring.
- Unfortunately hydrolysis of the peptidyl glycol racemic dye-linker **113** was only observed for one particular condition (additive cesium carbonate) and the level of hydrolysis was not very high.

Thus the two additives that had shown significant positive effects towards the hydrolysis of the peptidic systems were lithium iodide and cesium carbonate. Therefore in an attempt the level of hydrolysis, the peptidic glycol chiral dye-linker system **113** was then subjected to the two additives at different pH levels and also in dioxane as co-solvent. The results are summarised in Table 2.10 above.

It was found that when no additive or the additive lithium iodide which had caused the most hydrolysis to occur for the peptidyl glycol dye-linker system **101**, were applied to the chiral system **113** under the various new conditions, no hydrolysis was observed. However, when the additive cesium carbonate was

employed, hydrolysis was observed to occur for all cases. The conditions which gave the most hydrolysis were at pH 8 using acetonitrile as a co-solvent.

Regrettably there was not enough of the peptidyl glycol chiral dye-linker system **113** available to acquire enough of the hydrolysis product **108** to allow analysis *via* chiral HPLC. It was possible to conform by TLC (ethyl acetate:hexane) that the hydrolysis product was indeed **108**.

2.1.14 Conclusion and Future Recommendations

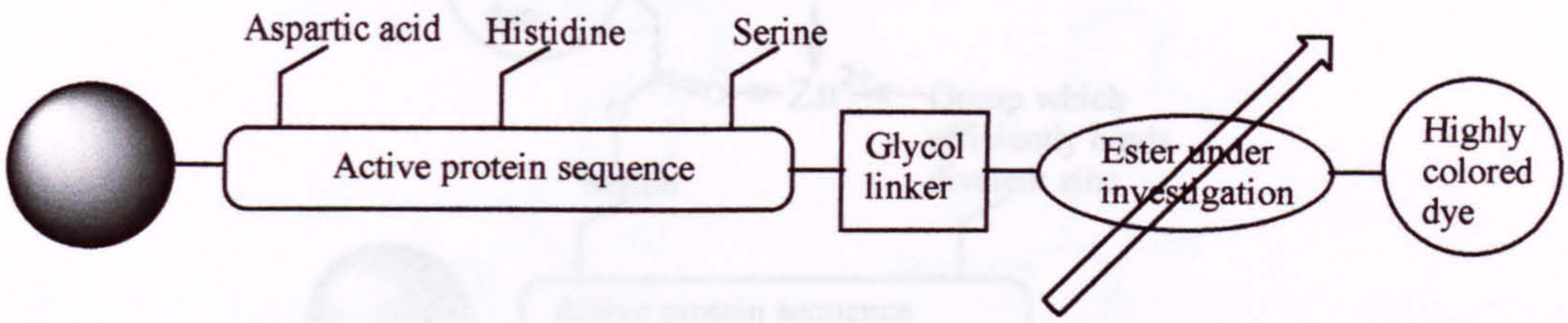
The synthesis of the model system allowing the investigation of intramolecular hydrolysis involving an ester tethered to a sixteen residue peptide through various lengthed glycol linkers was achieved. It was possible to show that the peptide appeared to be responsible for the intramolecular and intermolecular hydrolysis observed, and that the ester under investigation and the length of glycol linker employed, were important.

It was disappointing that the hydrolysis of the racemic peptidic glycol dye-linker system **113** could only be achieved under one set of conditions and this could have been due to the chiral center creating too much steric hindrance within the ester group for nucleophilic attack to occur. The glycol linker length employed for this system may not have been ideal. However, it was encouraging to observe that the hydrolysis seen in this example, was not observed within the

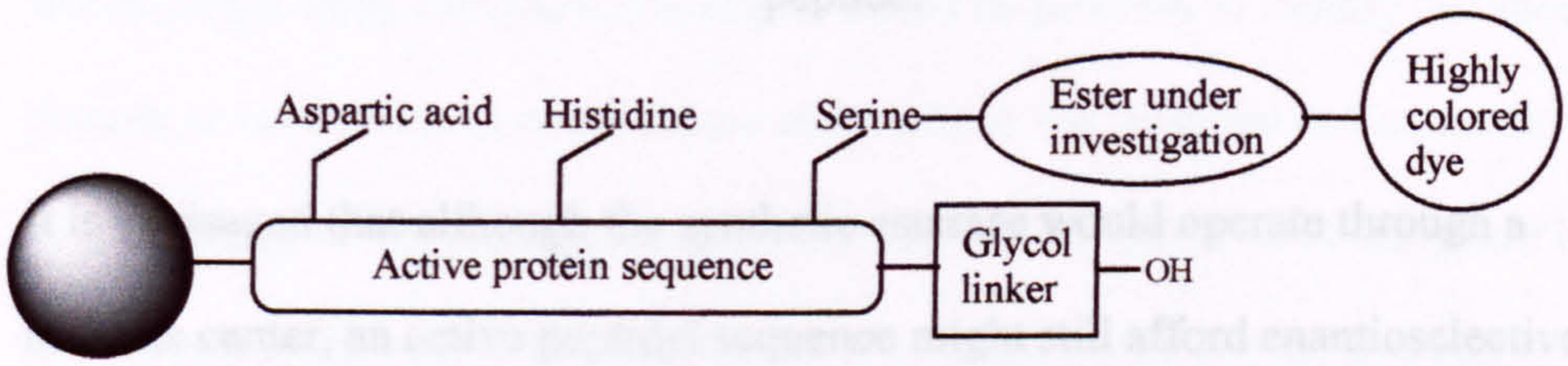
supported glycol dye-linker system **114** where no peptide was present, indicating the peptide was responsible for the hydrolysis.

It was observed during the hydrolysis experiments that the polymer support did not appear to swell noticeably once the glycol dye-linkers had been attached. Even with the most successful examples, significant levels of dye remained loaded on the support leaving a dark red resin. It is envisaged that utilising the PEGA gel resin, which is known to have a superior swelling capability to Tentagel described above, could give an improvement to the model system.

A further concern with the model system we have developed is that product inhibition may be occurring. Intramolecular cleavage of the ester within the glycol dye-linker component to form a new intermediate ester on the peptide may occur, however the new intermediate might be too stable to allow subsequent regeneration of the peptide *via* hydrolysis (Scheme 2.39).



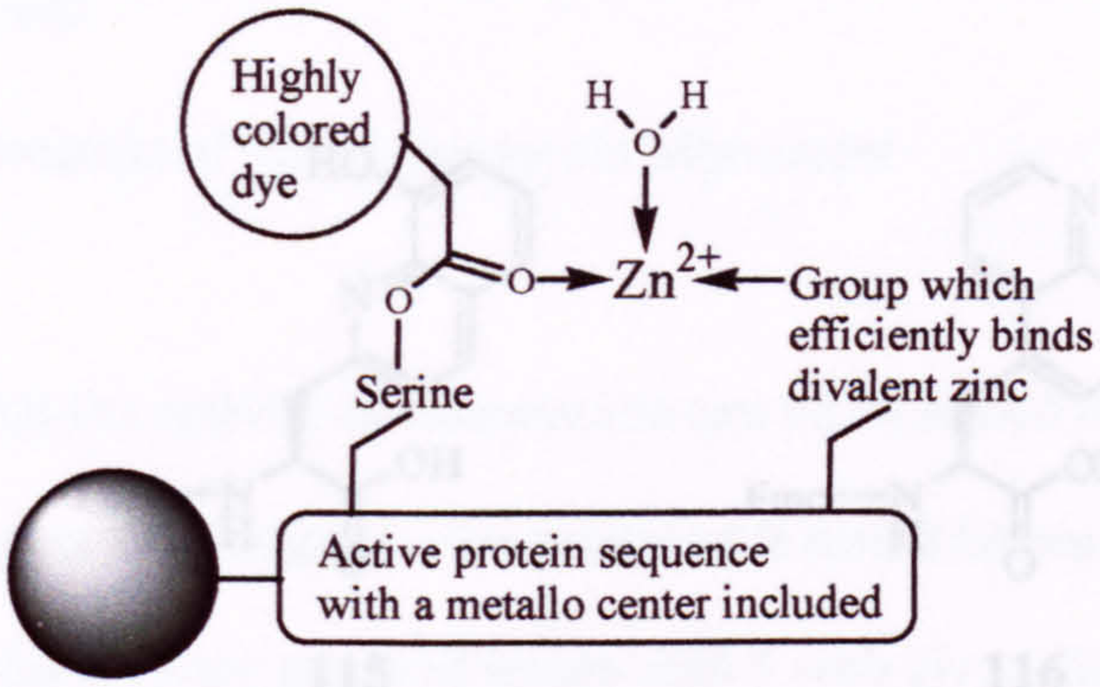
Initial intramolecular cleavage of the ester within the glycol dye-linker would give a peptidyl intermediate.



Subsequent hydrolysis of the intermediate releases the dye and regenerates the peptidyl system.

Scheme 2.39. Product inhibition.

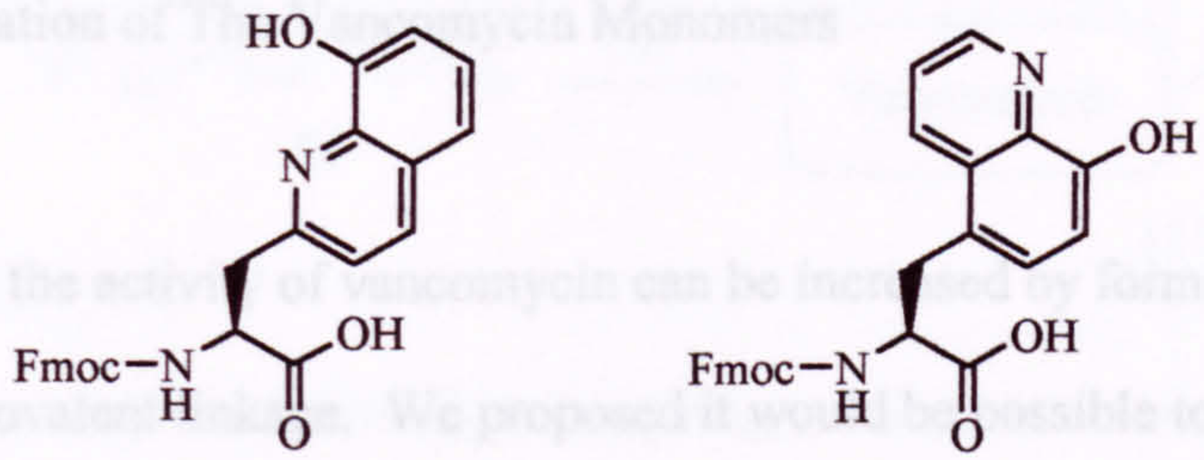
A possible modification to the peptidyl system could be the incorporation of a metallo center adjacent to the peptides active center. The function of the metallo center would be to bind a molecule of water in close proximity to assist the hydrolysis (Scheme 2.40).



Scheme 2.40. Incorporation of a metallo center in close proximity to the active center of the peptide may assist the hydrolysis and regeneration of the active peptide.

It is envisaged that although the synthetic esterase would operate through a metallic center, an active peptidyl sequence might still afford enantioselective control by providing a chiral environment around the active center.

A group to bind the metal ion could be introduced *via* unnatural bipyridyl amino acid residues[88] or residues designed by Barbara Imperiali *et al.*[89-91] who is interested in the development of fluorescent chemosensors. The incorporation of the unnatural amino acids (*S*)-2-amino-N-9-fluorenylmethoxycarbonyl-3-(oxine-2-yl) propionic acid **115** and (*S*)-2-amino-N-9-fluorenylmethoxycarbonyl-3-(oxine-5-yl) propionic acid **116** into short 7 residue peptides has shown that different metal ions can be selectively bound (Scheme 2.41). The selectivity is dependant on the amino acids incorporated in the seven residue peptide and is elucidated by observing the systems' fluorescence.



115 **116**

Scheme 2.41. Two unnatural amino acids incorporated in chemosensor systems.

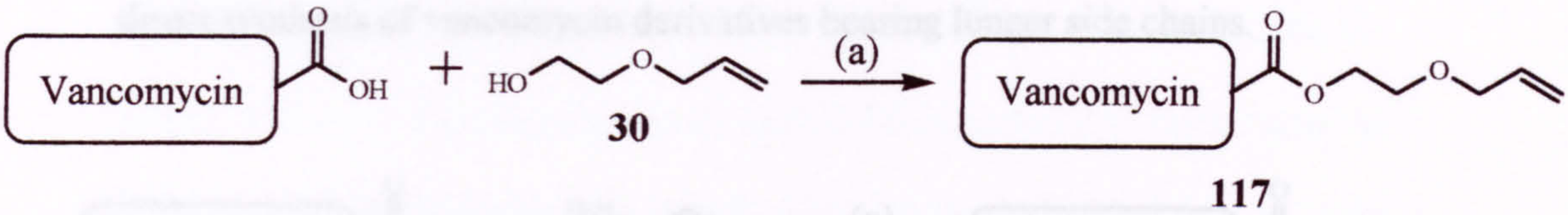
We envisage using the above strategy it would be possible to modify our model peptide to bind divalent zinc in close proximity to the potential active center. Tight binding of divalent zinc to our modified peptide could be investigated by fluorescent studies prior to attaching the glycol dye-linker component and undertaking the hydrolysis studies.

2.2 Vancomycin

2.2.1 Initial Formation of The Vancomycin Monomers

It was known that the activity of vancomycin can be increased by forming its dimer through a covalent linkage. We proposed it would be possible to modify vancomycin at the carboxy group of amino acid 7 with glycol linkers of varying lengths attached through an amide bond, bearing a terminal double bond to create a range of vancomycin monomers (Figure 1.14). It was envisaged that subjecting the vancomycin monomers to cross coupling metathesis (CCM) with Grubb's catalyst would yield a range of vancomycin dimers covalently tethered through different lengthed glycol linkers. A vancomycin dimer tethered through a glycol linker of an optimum length might show favourable activity against target bacteria which would be screened for *via* the ESI-MS technique developed by Professor Albert Heck described above.

The methodology for the synthesis of the vancomycin monomers was initially investigated by attempting to form an ester bond between vancomycin and 2-allyloxyethanol **30** *via* the coupling reagent PyBOP (Scheme 2.42). It was found upon analysis of the crude reaction mixture by reverse phase HPLC that a new species did form after 4 days although full conversion was not observed. The coupling was also attempted *via* Mitsunobu alkylation, however, the reverse phase HPLC analysis revealed the presence of several new products.

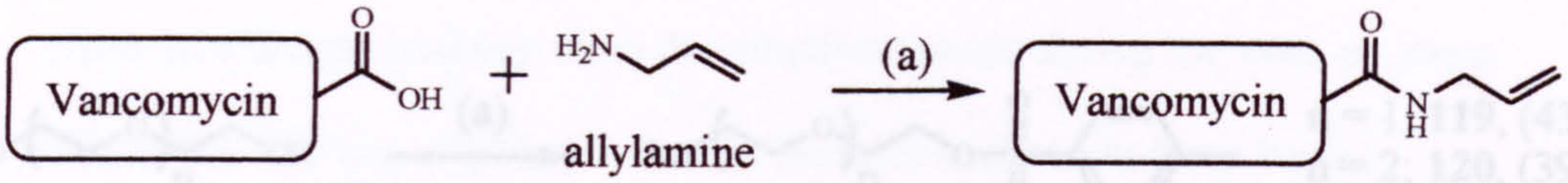


(a) Vancomycin hydrochloride (1.0 eq), **30** (5.0 eq), PyBOP, (2.5 eq), HOBt (2.5 eq), DIPEA (5.0 eq), room temperature, 5 days.

Scheme 2.42. Formation of vancomycin monomer **117** through an ester bond.

A difficulty encountered when handling vancomycin was that it was only sparingly soluble in most organic solvents including dimethylformamide. Solvents in which vancomycin did dissolve in well included; dimethyl sulfoxide, methanol and water. Therefore dimethyl sulfoxide was used as the reaction solvent when synthesising the vancomycin monomers. However, a drawback of dimethyl sulfoxide is that it has a high boiling point (189°C) and it was found that when attempting to remove the dimethyl sulfoxide under reduced pressure at the elevated temperature of 35°C, **117** decomposed. It was possible to remove dimethyl sulfoxide by freeze drying however due to the instability of **117** to moderately elevated temperatures, the synthesis of the vancomycin monomers through an amide, than an ester, bond was investigated (Scheme 2.43). The coupling of allylamine to vancomycin was undertaken *via* the coupling reagent PyBOP with dimethyl sulfoxide as solvent. It was found that a new product **118** was formed and the rate of reaction appeared by reverse phase HPLC analysis, to be greater than for the ester formation. The product was not isolated, however it was found to be stable at the elevated temperature of 50°C. Indeed, it was

decided that a more productive course of action would be to move on to the direct synthesis of vancomycin derivatives bearing longer side chains.



118

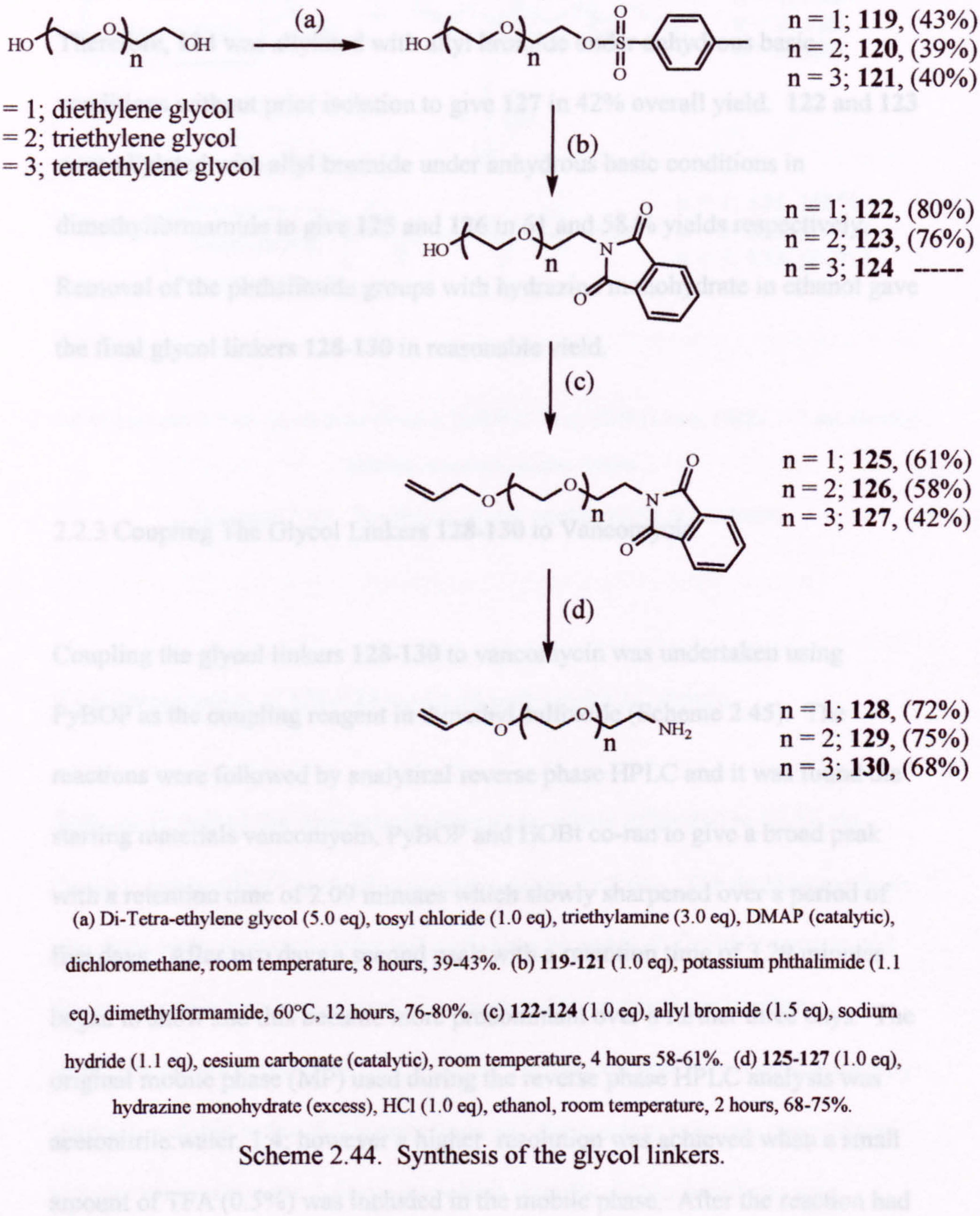
(a) Vancomycin hydrochloride (1.0 eq), allylamine (5.0 eq), PyBOP, (2.5 eq), HOBt (2.5 eq), DIPEA (5.0 eq), room temperature, 3 days.

Scheme 2.43. Formation of vancomycin monomer **118** through an amide bond.

2.2.2 Synthesis of The Glycol Linkers

The linkers used to modify vancomycin were synthesised from di, tri and tetraethylene glycol (Scheme 2.44). Modification of the ethylene glycol starting materials was initially attempted by forming the mono-allyl derivatives under anhydrous basic conditions in tetrahydrofuran using allyl bromide. A five fold excess of the ethylene glycol was used in each case to reduce the formation of the unwanted bis-allyl product. However, it was observed by TLC (ethyl acetate:hexane, 3:2) that in each case the bis-allyl product predominated. It appeared that the mono-allyl products were more reactive during the allylation conditions than their corresponding ethylene glycol starting materials. Therefore, due to the difficulty of forming the mono-allyl products, the mono-tosyl derivatives **119-121** were synthesised. An excess of the ethylene glycol

starting materials was used and the mono-tosylate derivatives were obtained in moderate yields of 39-43%.



(a) Di-Tetra-ethylene glycol (5.0 eq), tosyl chloride (1.0 eq), triethylamine (3.0 eq), DMAP (catalytic), dichloromethane, room temperature, 8 hours, 39-43%. (b) **119-121** (1.0 eq), potassium phthalimide (1.1 eq), dimethylformamide, 60°C, 12 hours, 76-80%. (c) **122-124** (1.0 eq), allyl bromide (1.5 eq), sodium hydride (1.1 eq), cesium carbonate (catalytic), room temperature, 4 hours 58-61%. (d) **125-127** (1.0 eq), hydrazine monohydrate (excess), HCl (1.0 eq), ethanol, room temperature, 2 hours, 68-75%.

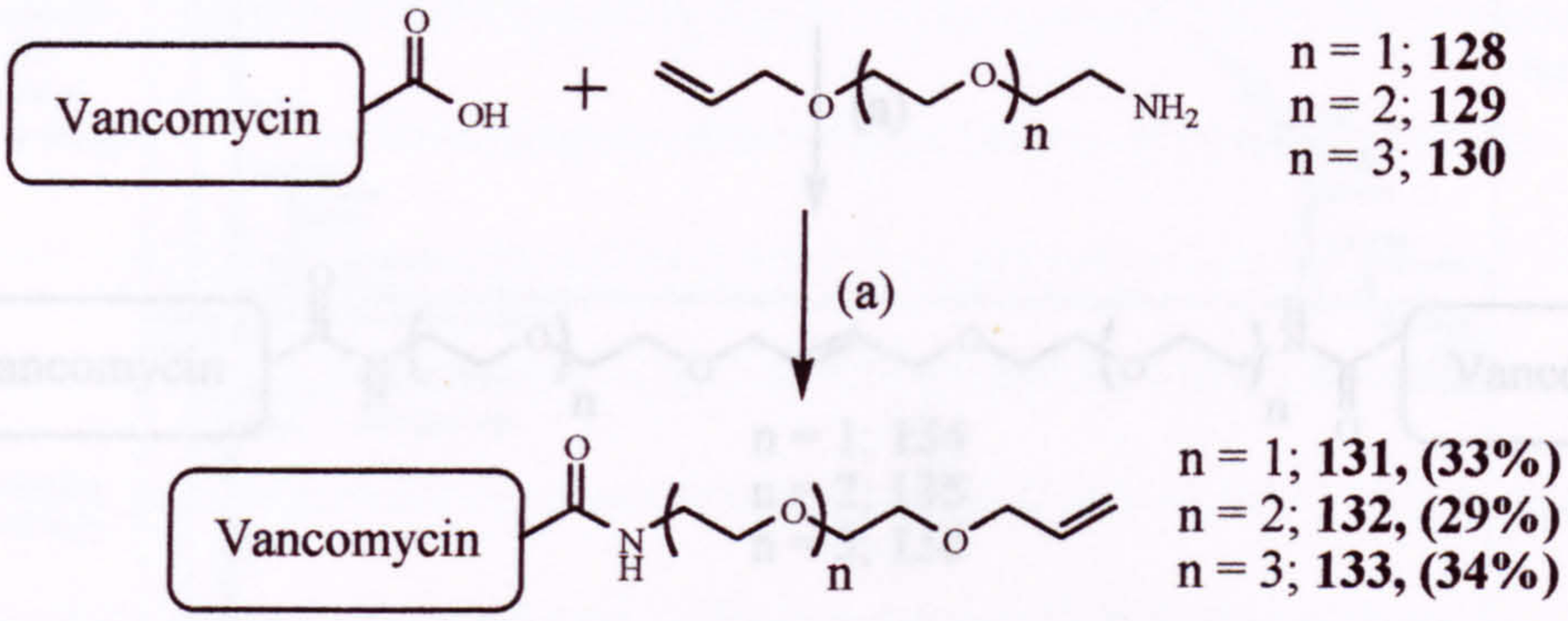
Scheme 2.44. Synthesis of the glycol linkers.

The displacement of the tosylate groups by potassium phthalimide could be achieved in reasonable yields (76-80%) in dimethylformamide at 60°C for 12 hours. However, the tetraethylene derivative **124** was found to be too polar to allow its efficient isolation from dimethylformamide during the work up stage. Therefore, **124** was allylated with allyl bromide under anhydrous basic conditions without prior isolation to give **127** in 42% overall yield. **122** and **123** were allylated with allyl bromide under anhydrous basic conditions in dimethylformamide to give **125** and **126** in 61 and 58 % yields respectively. Removal of the phthalimide groups with hydrazine monohydrate in ethanol gave the final glycol linkers **128-130** in reasonable yield.

2.2.3 Coupling The Glycol Linkers **128-130** to Vancomycin

Coupling the glycol linkers **128-130** to vancomycin was undertaken using PyBOP as the coupling reagent in dimethyl sulfoxide (Scheme 2.45). The reactions were followed by analytical reverse phase HPLC and it was found the starting materials vancomycin, PyBOP and HOBt co-ran to give a broad peak with a retention time of 2.09 minutes which slowly sharpened over a period of five days. After two days a second peak with a retention time of 3.29 minutes began to show and this became more predominant over a further three days. The original mobile phase (MP) used during the reverse phase HPLC analysis was acetonitrile:water, 1:4; however a higher resolution was achieved when a small amount of TFA (0.5%) was included in the mobile phase. After the reaction had been stirred at room temperature for 5 days the dimethyl sulfoxide was removed

by freeze drying and the vancomycin monomers were isolated by preparative reverse phase HPLC to give **131-133** in 29-34% yield.

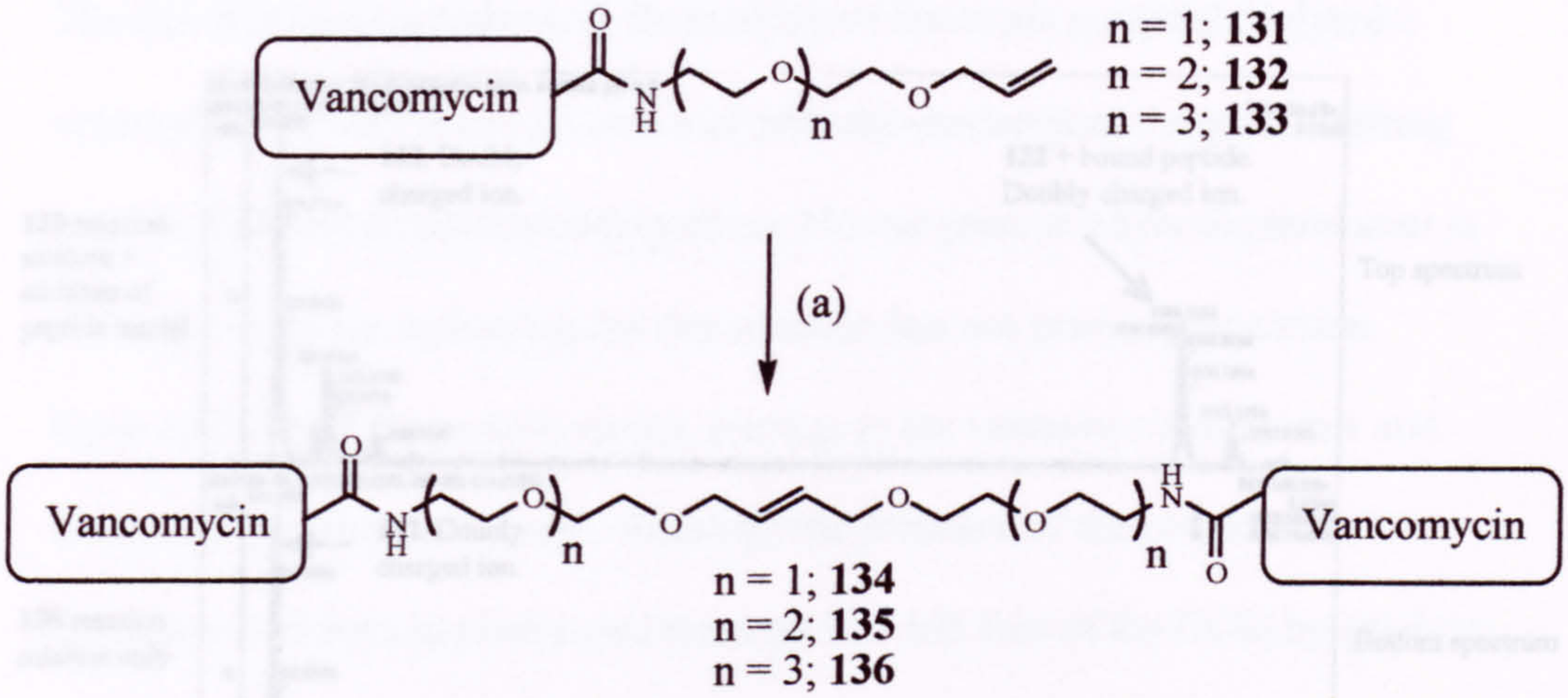


(a) Vancomycin (1.0 eq), glycol linker (excess), PyBOP (1.2 eq), HOBt (1.2 eq), DIPEA (1.2 eq), dimethyl sulfoxide, room temperature, 4 days.

Scheme 2.45. Synthesis of the vancomycin monomers.

2.2.4 Dimerisation of **131-133** *via* CCM with Grubb's Catalyst

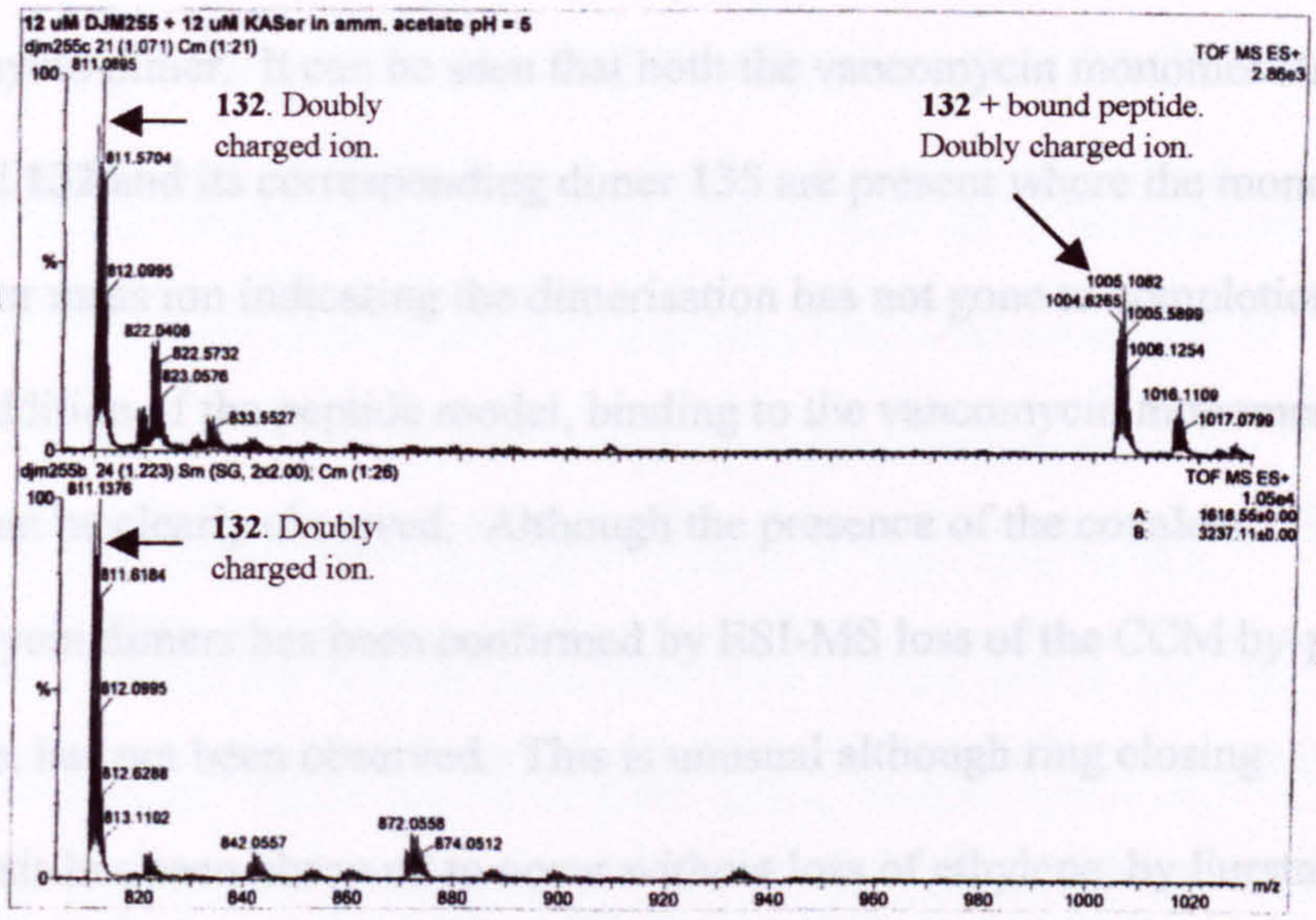
Samples (1 mg) of the vancomycin monomers where subjected to CCM *via* Grubb's catalyst[92,93] in dichloromethane (Scheme 2.46). The dichloromethane was previously degassed under reduced pressure and both Grubb's catalyst and the vancomycin monomers were dissolved in dichloromethane by ultra sonification over a period of ten minutes. The vancomycin monomers were only sparingly soluble in the dichloromethane and the reaction mixtures were stirred at room temperature for 2 days after which time the dichloromethane was removed under reduced pressure.



(a) Vancomycin monomer (1 eq), Grubb's catalyst (25 mol%), dichloromethane, room temperature, 2 days.

Scheme 2.46. Dimerisation *via* CCM with Grubb's catalyst.

Samples of the vancomycin monomers **131-133** and their corresponding potential dimers **134-136** were sent to Professor Albert Heck in Utrecht for ESI-MS analysis and the vancomycin derivatives have been demonstrated to bind *N*-acetyl-L-Lys-D-Ala-D-Ser (Picture 2.2). The model peptide *N*-acetyl-L-Lys-D-Ala-D-Ser was chosen for the initial binding studies as this represents a strain of bacteria whose pentapeptide has shown resistance to vancomycin, as described above.

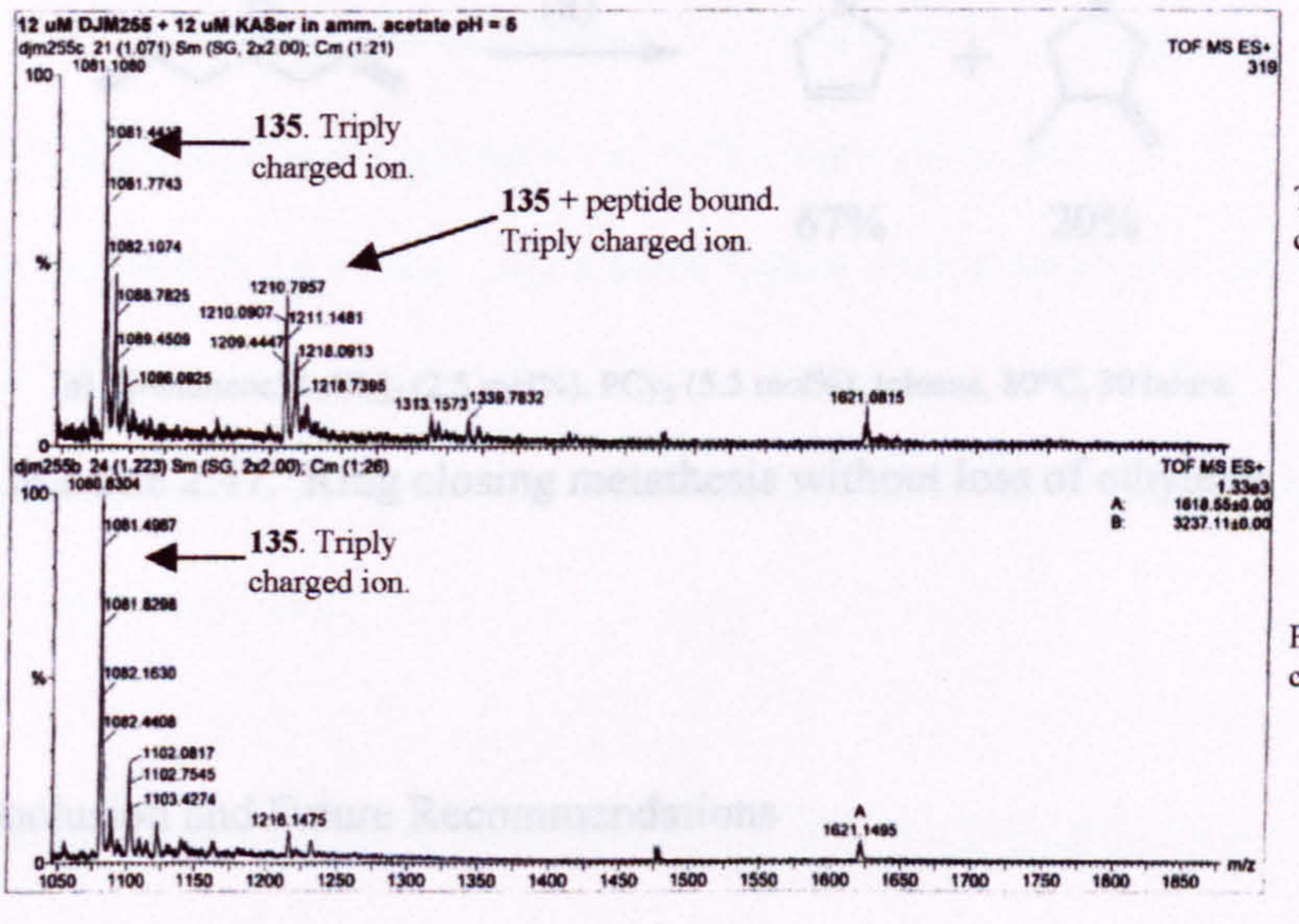


135 reaction mixture + addition of peptide model

Top spectrum

135 reaction mixture only

Bottom spectrum



135 reaction mixture + addition of peptide model

Top spectrum continued

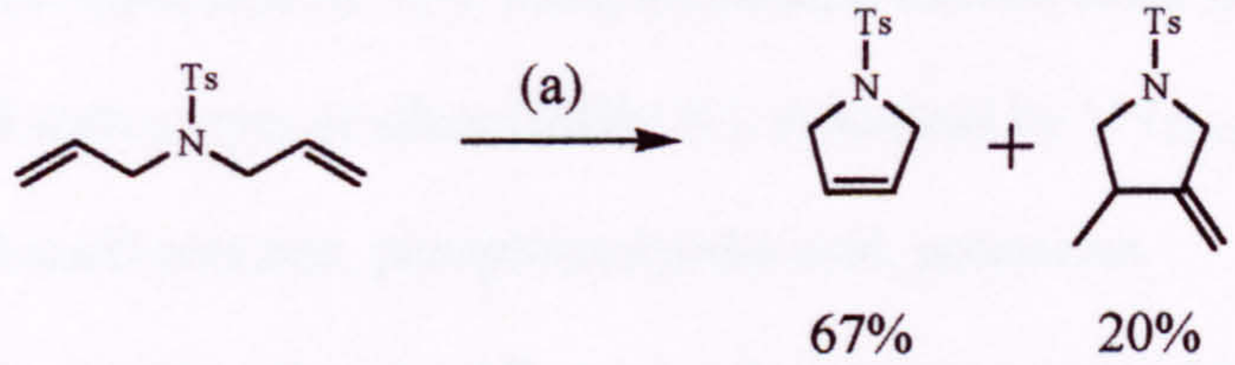
135 reaction mixture only

Bottom spectrum continued

Picture 2.2. The ESI-MS for **135** is shown in the bottom spectrum. The ESI-MS for **135** and binding with the *N*-acetyl-L-Lys-D-Ala-D-Ser model peptide is shown in the top spectrum.

The ESI-MS spectrum above is the analysis of the crude potential triglycol vancomycin dimer. It can be seen that both the vancomycin monomer starting material **132** and its corresponding dimer **135** are present where the monomer is the major mass ion indicating the dimerisation has not gone to completion.

Upon addition of the peptide model, binding to the vancomycin monomer and dimer can be clearly observed. Although the presence of the covalent vancomycin dimers has been confirmed by ESI-MS loss of the CCM by-product, ethylene, has not been observed. This is unusual although ring closing metathesis has been observed to occur without loss of ethylene, by Furstner *et al.*[94] (Scheme 2.47).



(a) $[(p\text{-cumene})RuCl_2]_2$ (2.5 mol%), PCy_3 (5.5 mol%), toluene, 80°C, 30 hours.

Scheme 2.47. Ring closing metathesis without loss of ethylene.

2.2.5 Conclusion and Future Recommendations

A range of vancomycin monomers have been successfully synthesised and shown to bind *N*-acetyl-L-Lys-D-Ala-D-Ser. The dimerisation of the vancomycin monomers *via* CCM has been attempted with Grubb's catalyst and the presence and the ability of vancomycin dimers to bind *N*-acetyl-L-Lys-D-Ala-D-Ser has

been shown. However, further work is required to indicate that the vancomycin monomers have indeed formed covalently linked dimers, or if the dimers are spontaneously forming in solution. Other catalysts to afford the CCM of the vancomycin monomers shall also be investigated to encourage a higher conversion than previously observed.

The vancomycin dimers are to be isolated by preparative reverse phase HPLC and *in vivo* studies are to be undertaken for both the potential vancomycin dimers and their corresponding monomers, to establish if enhanced activity can be observed. Studies are also ongoing to elucidate the binding efficiencies of the vancomycin monomers and dimers to *N*-acetyl-L-Lys-D-Ala-D-Ser and other peptide model systems.

3. Experimental

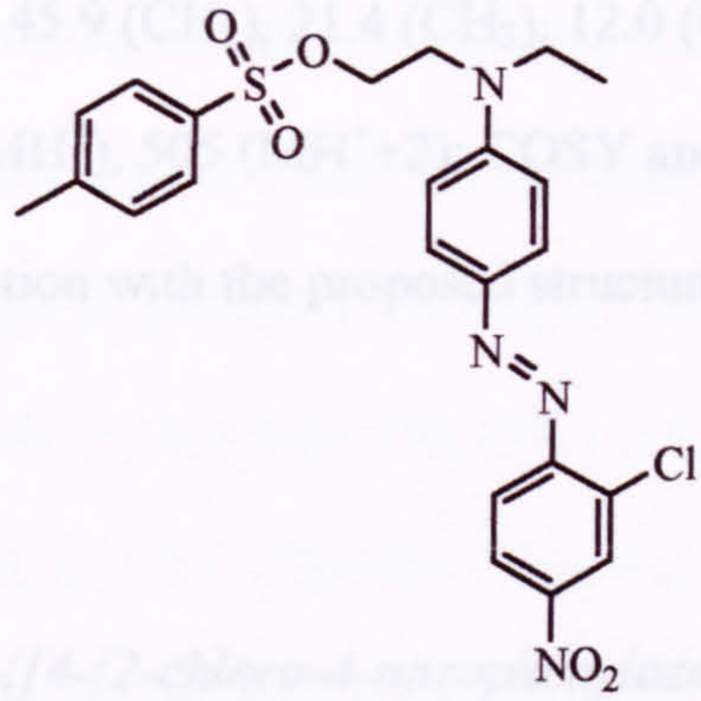
All air and moisture sensitive reactions were performed under an atmosphere of dry nitrogen in flame dried glassware. All anhydrous solvents were used as supplied by Romil in HyDry™ bottles except dimethyl sulfoxide and dimethylformamide which were supplied by Aldrich in Sure Seal bottles. Commercially available starting materials were used without further purification unless otherwise stated. Petroleum ether refers to that fraction which boils in the range 40-60 °C and $MgSO_4$ was used as the drying agent for solutions.

The reactions were monitored by TLC using aluminium backed silica gel (F_{254}) plates, pre-coated with a layer of silica (MERCK), visualised by UV_{254nm} and then 2,4-dinitrophenolhydrazine, phosphomolybdic acid, potassium permanganate or ninhydrin solution. All organic layers were removed by rotary evaporation on a Büchi rotary evaporator, the final traces of solvents being removed on a static oil pump (0.2 mbar). Column chromatography was carried out on silica gel 60 (40-63 μm).

Melting points were measured on a Stuart Scientific SMP1 instrument and are uncorrected. Infrared spectra (IR) were recorded on a Perkin Elmer 1310 FTIR spectrometer between sodium chloride plates. Nuclear magnetic resonance spectra (NMR) were recorded on either a Brucker AC 250 MHz or 300 MHz spectrometer. The chemical shift values were quoted in values of ppm and these were relative to the standard tetramethylsilane (TMS) for ^1H NMR and to the

centre line of the chloroform triplet, δ 77.0, for ^{13}C NMR. The peak multiplicities were specified as singlet (s), doublet (d), doublet-doublet (dd), triplet (t), quartet (q) or quintet (quint) and coupling constants (*J*) quoted in Hz. For all AA'BB' aromatic systems, the multiplicity is quoted as d with the coupling constant quoted as J_{AB}. Mass Spectra (MS) were obtained from the EPSRC Mass Spectrometry Service Centre, Swansea, as were high resolution determinations unless indicated, then Mass Spectra were obtained with a Kratos analytical MS80 RFAO spectrometer. Elemental analyses were performed with a Carlo Erba 1106 elemental analyser. Reverse phase HPLC was performed with a C_{18} Anachem ODS2 analytical column or a C_{18} technology ODS semi-preparative column using a Krontron HPLC pump 422 and 332 UV detector. The absorbance was measure at 254 nm.

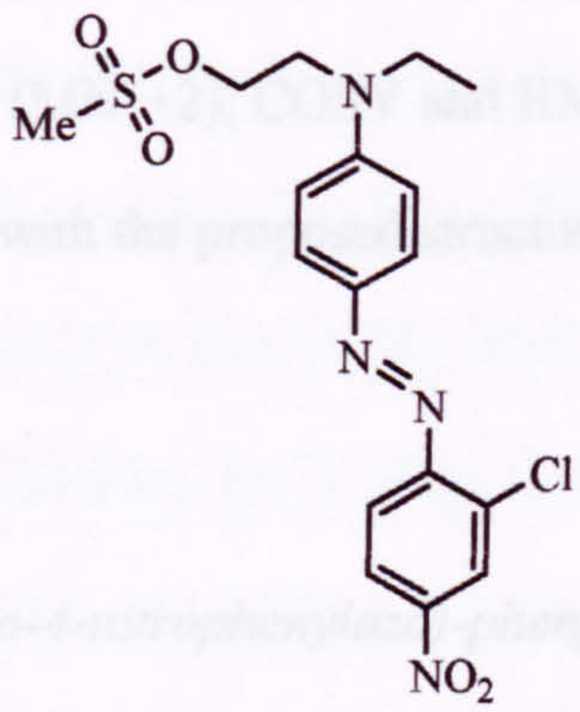
Toluene-4-sulfonic acid 2-{[4-(2-chloro-4-nitrophenylazo)-phenyl]-ethylamino}-ethyl ester, **16**.[45]



To a solution of disperse red (3 g, 8.6 mmol) in dichloromethane (40 ml) was added triethylamine (3.6 ml, 25.8 mmol, 3 eq) and *p*-toluene sulfonyl chloride (3.6 g, 18.9 mmol, 2.2 eq) at 0°C. The reaction mixture was then stirred at room temperature for 12 hours after which time TLC (ethyl acetate:hexane, 4:1) indicated that the starting material had been consumed. The organic solution was then washed with water (3 x 30 ml), the organic extract dried ($MgSO_4$) and concentrated in *vacuo*. The product was eluted by flash column chromatography (ethyl acetate:hexane, 2:3) to give a reddish brown powder. The product was further purified by recrystallisation using ethyl acetate/hexane to give **16** as dark metallic green elongated needles, (3.2 g, 6.4 mmol, 74%). mp 127-129°C (ethyl acetate/hexane); (Found: C, 54.78; H, 4.60; N, 11.05. $C_{23}H_{23}ClN_4O_5S$ requires C, 54.92; H, 4.61; N, 11.14%); ν_{max}(Nujol)/cm^{-1} 1598, 1463, 1338; δ_{II}(300 MHz; $CDCl_3$) 8.35 (1 H, d, *J* 2.4, ArC**H**), 8.12 (1 H, dd, *J* 8.9, 2.4, ArC**H**), 7.85 (2 H, d, J_{AB} 9.2, ArC**H**), 7.75 (1 H, d, *J* 8.9, ArC**H**), 7.73 (2 H, d, J_{AB} 8.2, ArC**H**), 7.27 (2 H, d, J_{AB} 8.2, ArC**H**), 6.61 (2 H, d, J_{AB} 9.2, ArC**H**), 4.22 (2 H, t, *J* 5.9, TsOC**H**$_2$CH$_2$), 3.71 (2 H, t, *J* 5.9, TsOCH$_2$C**H**$_2$), 3.44 (2 H, q, *J* 7.1, C**H**$_2$Me), 2.39 (3 H, s, Ar**Me**), 1.19 (3 H, t, *J* 7.1, CH$_2$**Me**); δ_C(75 MHz; $CDCl_3$) 152.7 (C),

150.8 (C), 147.0 (C), 145.0 (C), 144.3 (C), 133.9 (C), 132.2 (C), 129.7 (2CH), 127.6 (2CH), 126.6 (CH), 125.8 (CH), 122.4 (2CH), 117.8 (CH), 111.2 (2CH), 66.2 (CH_2), 48.8 (CH_2), 45.9 (CH_2), 21.4 (CH_3), 12.0 (CH_3); *m/z* (EI) 502 (M^+), 504 (M^++2); (CI) 503 (MH^+), 505 (MH^++2); COSY and HMQC spectra both exhibited a good correlation with the proposed structure.

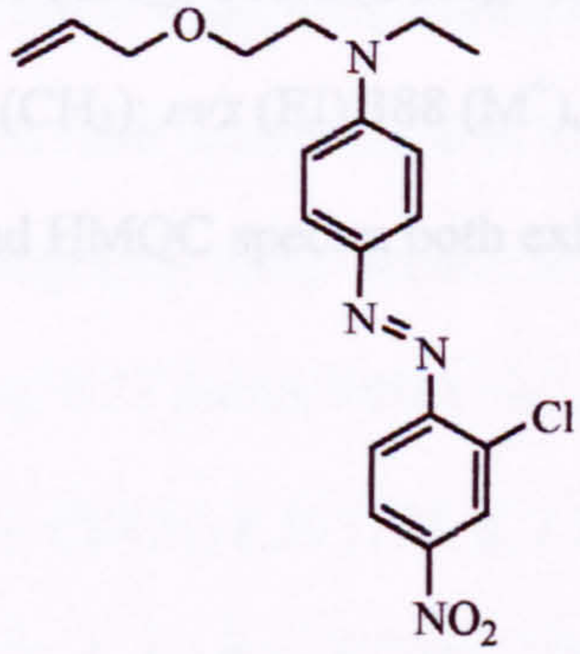
Methanesulfonic acid 2-{[4-(2-chloro-4-nitrophenylazo)-phenyl]-ethylamino}-ethyl ester, **17**.[46]



To a solution of disperse red (3 g, 8.6 mmol) in dichloromethane (40 ml) was added triethylamine (3.6 ml, 25.8 mmol, 3 eq) and methane sulphonyl chloride (1.48 ml, 18.9 mmol, 2.2 eq) at 0°C. The reaction mixture was then stirred at room temperature for 2 hours after which time TLC (ethyl acetate:hexane, 4:1) indicated that the starting material had been consumed. The organic solution was then washed with water (3 x 30 ml), the organic extract dried ($MgSO_4$) and concentrated in *vacuo*. The product was eluted by flash column chromatography (ethyl acetate:hexane, 2:3) to give a dark red powder. The product was further purified by recrystallisation using ethyl acetate/hexane to give **17** as dark

metallic green elongated needles, (2.94 g, 6.89 mmol, 80%). mp 138-139°C (ethyl acetate/hexane); (Found: C, 47.83; H, 4.47; N, 13.15. $C_{17}H_{19}ClN_4O_5S$ requires C, 47.83; H, 4.49; N, 13.12%); ν_{max}(Nujol)/cm^{-1} 1600, 1516, 1332; δ_H(300 MHz; $CDCl_3$) 8.32 (1 H, d, *J* 2.5, ArC**H**), 8.09 (1 H, dd, *J* 9.0, 2.5, ArC**H**), 7.90 (2 H, d, J_{AB} 9.2, ArC**H**), 7.72 (1 H, d, *J* 9.0, ArC**H**), 6.77 (2 H, d, J_{AB} 9.2, ArC**H**), 4.42 (2 H, t, *J* 6.0, MsOC**H**$_2$CH$_2$), 3.81 (2 H, t, *J* 6.0, MsOCH$_2$C**H**$_2$), 3.56 (2 H, q, *J* 7.1, C**H**$_2$Me), 3.02 (3 H, s, Ms**Me**), 1.27 (3 H, t, *J* 7.1, CH$_2$**Me**); δ_C(75 MHz; $CDCl_3$) 153.2 (C), 151.6 (C), 147.6 (C), 145.0 (C), 134.5 (C), 127.3 (CH), 126.4 (CH), 123.0 (CH), 118.4 (2CH), 112.0 (2CH), 66.4 (CH_2), 49.8 (CH_2), 46.5 (CH_2), 38.0 (CH_3), 12.7 (CH_3); *m/z* (EI) 426 (M^+), 428 (M^++2); (C) 427 (MH^+), 429 (MH^++2); COSY and HMQC spectra both exhibited a good correlation with the proposed structure.

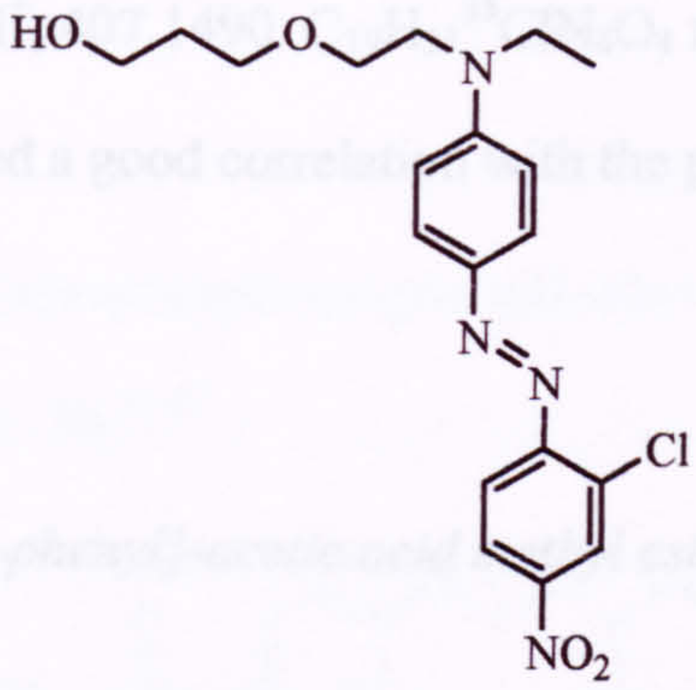
(2-Allyloxyethyl)-[4-(2-chloro-4-nitrophenylazo)-phenyl]-ethylamine, **22**.



To a solution of disperse red (1.0 g, 2.87 mmol) dissolved in dimethylformamide (10 ml) was carefully added cesium carbonate (100 mg, catalytic), sodium hydride [50% w/w dispersion in mineral oil], (276 mg, 5.75 mmol, 2.0 eq) and allyl bromide (0.37 ml, 4.31 mmol, 1.5 eq) at 0°C. The reaction mixture was

stirred at room temperature for 4 hours after which time TLC (ethyl acetate:hexane, 4:1) indicated that the starting material had been consumed. The solution was quenched with water, diluted with diethyl ether (40 ml), washed with water (3 x 30 ml), dried ($MgSO_4$), and concentrated in *vacuo*. The product was eluted by flash column chromatography (ethyl acetate:hexane, 1:4) to give **22** as a dark metallic green oil, (1.08 g, 2.78 mmol, 97%). mp 51-52°C (ethyl acetate/hexane); (Found: C, 58.55; H, 5.38; N, 14.47. $C_{19}H_{21}ClN_4O_3$ requires C, 58.69; H, 5.44; N, 14.41%); ν_{max}(Nujol)/cm^{-1} 1602, 1511, 1337; δ_H(300 MHz; $CDCl_3$) 8.32 (1 H, d, *J* 2.5, ArC**H**), 8.08 (1 H, dd, *J* 9.1, 2.5, ArC**H**), 7.88 (2 H, d, J_{AB} 9.2, ArC**H**), 7.73 (1 H, d, *J* 9.1, ArC**H**), 6.74 (2 H, d, J_{AB} 9.2, ArC**H**), 5.90 (1 H, ddt, *J* 17.1, 10.4, 5.5, $CH_2C\mathbf{H}CH_2O$), 5.27 (1 H, ddt, *J* 17.1, 1.5, 1.5, *trans*-$C\mathbf{H}_2CHCH_2O$), 5.19 (1 H, ddt, *J* 10.4, 1.3, 1.3, *cis*-$C\mathbf{H}_2CHCH_2O$), 4.01 (2 H, dt, *J* 5.5, 1.3, $CH_2CHC\mathbf{H}_2O$), 3.64 (4 H, br t, $OC\mathbf{H}_2C\mathbf{H}_2N$), 3.54 (2 H, q, *J* 7.1, $C\mathbf{H}_2Me$), 1.24 (3 H, t, *J* 7.1, $CH_2\mathbf{Me}$); δ_C(75 MHz; $CDCl_3$) 153.5 (C), 152.3 (C), 147.3 (C), 144.6 (C), 134.8 (CH), 134.2 (C), 127.4 (CH), 126.3 (CH), 123.0 (2CH), 118.3 (CH), 117.6 (CH_2), 111.8 (2CH), 72.7 (CH_2), 68.0 (CH_2), 50.8 (CH_2), 46.5 (CH_2), 12.6 (CH_3); *m/z* (EI) 388 (M^+), 390 (M^++2); (CI) 389 (MH^+), 391 (MH^++2); COSY and HMQC spectra both exhibited a good correlation with the proposed structure.

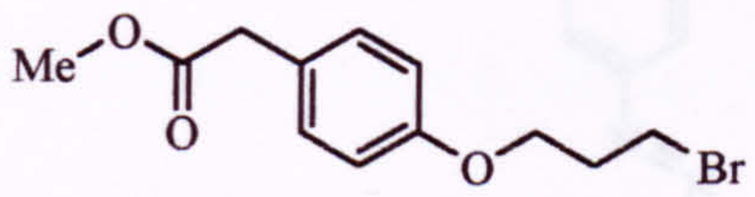
3-(2-{[4-(2-Chloro-4-nitrophenylazo)-phenyl]-ethylamino}-ethoxy)-propan-1-ol, **23**.[48]



To a solution of the allyl derivative of disperse red **22** (200 mg, 0.57 mmol) in toluene (1 ml) was added borane methyl sulfide (2 M in toluene, 0.29 ml, 0.57 mmol) at 0°C. The reaction mixture was stirred at room temperature for 3 hours after which time TLC (ethyl acetate:hexane, 2:3) indicated that the starting material had been consumed. This was then quenched carefully at 0°C with methanol (2 ml), then hydrogen peroxide (30% aq. 0.13 ml, 1.15 mmol, 2 eq) and sodium hydroxide (2 M, 0.72 ml, 1.43 mmol, 2.5 eq) were carefully added. The reaction mixture was stirred at room temperature for 2 hours, washed with water (3 x 40 ml), dried ($MgSO_4$) and concentrated in *vacuo*. The product was eluted by flash column chromatography (ethyl acetate:hexane, 1:4) to give **23** as a dark metallic green oil, (93 mg, 0.23 mmol, 40%). ν_{max}(Nujol)/cm^{-1} 3384, 1600, 1517, 1336; δ_H(300 MHz; $CDCl_3$) 8.39 (1 H, d, *J* 2.4, ArC**H**), 8.16 (1 H, dd, *J* 9.0, 2.4, ArC**H**), 7.94 (2 H, d, J_{AB} 9.2, ArC**H**), 7.78 (1 H, d, *J* 9.0, ArC**H**), 6.78 (2 H, d, J_{AB} 9.2, ArC**H**), 3.77 (2 H, dt, *J* 5.6, HOC**H**$_2$$CH_2CH_2$), 3.69-3.62 (6 H, m, C**H**$_2$), 3.56 (2 H, q, *J* 7.2, C**H**$_2$Me), 1.99 (1 H, t, *J* 5.6, **H**O$CH_2CH_2CH_2$), 1.85 (2 H, quint, *J* 5.6, HOCH_2C**H**$_2$$CH_2$), 1.25 (3 H, t, *J* 7.2, CH_2**Me**); δ_C(75 MHz; $CDCl_3$) 153.5 (C), 152.3 (C), 147.4 (C), 144.6 (C), 134.3 (C), 127.4 (CH), 126.4

(CH), 123.0 (2CH), 118.4 (CH), 111.8 (2CH), 70.7 (CH_2), 69.0 (CH_2), 61.9 (CH_2), 50.7 (CH_2), 46.5 (CH_2), 32.5 (CH_2), 12.6 (CH_3); *m/z* (CI) 407 (MH^+), 409 (MH^++2); (Found: MH^+, 407.1490. $C_{19}H_{23}{}^{35}ClN_4O_4$ requires MH^+, 407.1486). COSY spectra exhibited a good correlation with the proposed structure.

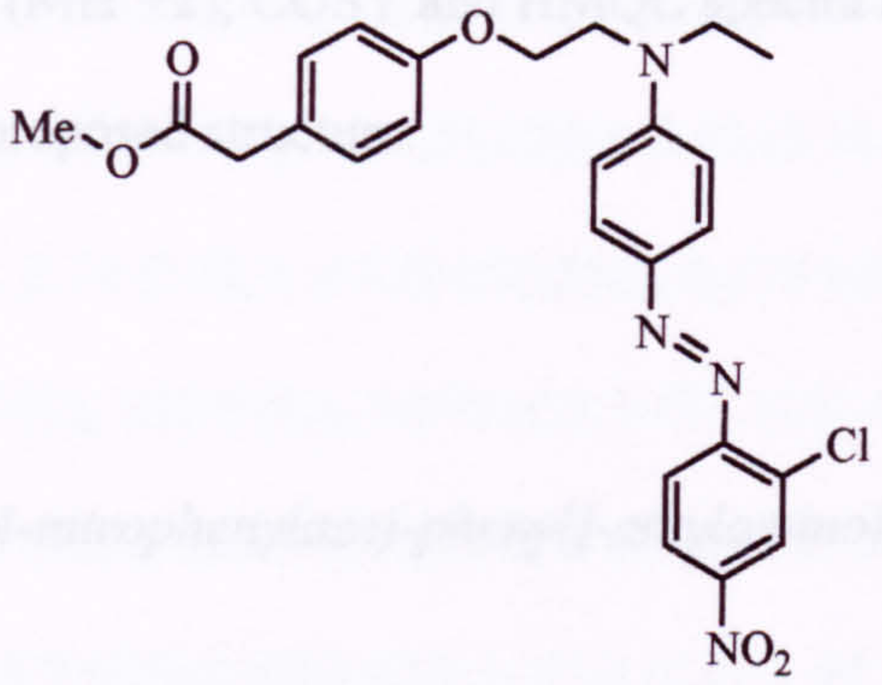
[4-(3-Bromo-propoxy)-phenyl]-acetic acid methyl ester, **19**.



To a solution of methyl 4-hydroxyphenyl acetate (5.0 g, 30 mmol), in tetrahydrofuran (20 ml) was added 1,3-dibromopropane (6.1 ml, 60 mmol, 2 eq) and sodium hydride [50% w/w dispersion in mineral oil], (1.4 g, 30 mmol). The reaction mixture was stirred at room temperature for 12 hours after which time TLC (ethyl acetate:hexane, 1:4) indicated that the starting material had been consumed. This was then diluted with diethyl ether (30 ml), washed with water (3 x 30 ml), dried ($MgSO_4$) and concentrated in *vacuo*. The product was isolated by flash column chromatography (ethyl acetate:hexane, 1:4) to give **19** as a clear colourless oil, (3.6 g, 12.5 mmol, 42%). (Found: C, 50.27; H, 5.26. $C_{12}H_{15}BrO_3$ requires C, 50.19; H, 5.27%); ν_{max}(Nujol)/cm^{-1} 1736, 1611; δ_H(300 MHz; $CDCl_3$) 7.19 (2 H, d, J_{AB} 8.7, ArC**H**), 6.86 (2 H, d, J_{AB} 8.7, ArC**H**), 4.07 (2 H, t, *J* 6.5, $CH_2CH_2C\mathbf{H}_2Br$), 3.67 (2 H, s, ArC$\mathbf{H}_2$) 3.59 (2 H, t, *J* 6.5, $C\mathbf{H}_2CH_2CH_2Br$), 3.56 (3 H, s, $CO_2\mathbf{Me}$), 2.29 (2 H, quint, *J* 6.5, $CH_2C\mathbf{H}_2CH_2Br$); δ_C(75 MHz; $CDCl_3$) 172.7 (C), 158.2 (C), 130.7 (2CH), 126.7 (C), 115.2 (2CH),

65.7 (CH_2), 52.4 (CH_3), 40.7 (CH_2), 32.7 (CH_2), 30.5 (CH_2); *m/z* (EI) 286 (M^+), 288 (M^++2); (CI) 181 (MH^+), 198 (MNH_4^+).

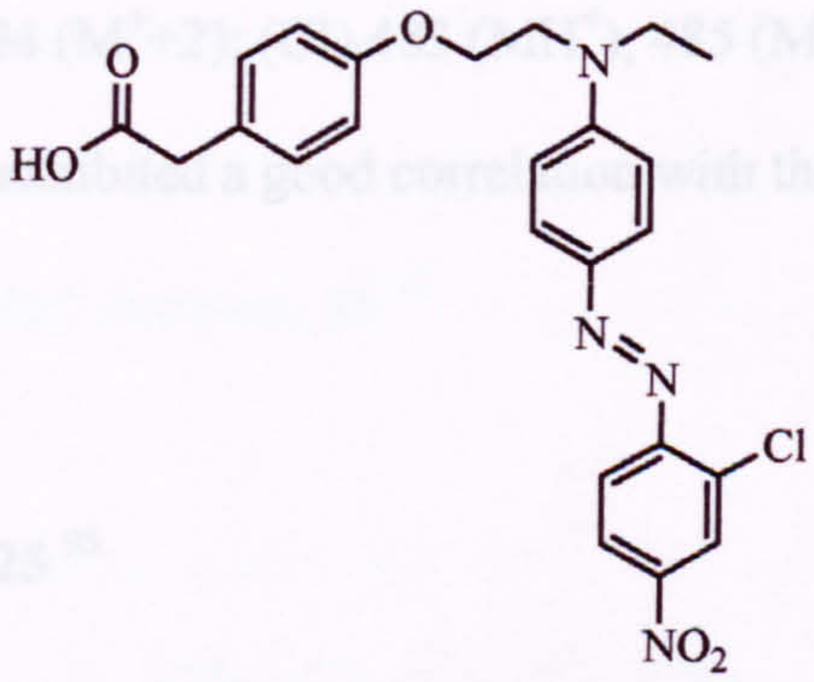
[4-(2-{[4-(2-Chloro-4-nitrophenylazo)-phenyl]-ethylamino}-ethoxy)-phenyl]-acetic acid methyl ester, **18**.[55-57]



To a solution of methyl 4-hydroxyphenyl acetate (4.8 g, 29 mmol), triphenylphosphine (7.6 g, 29 mmol) and diethyl azodicarboxylate (4.6 ml, 29 mmol) in toluene (350 ml) at 80°C was added disperse red (5 g, 14.3 mmol, 0.5 eq). The reaction mixture was stirred at 80°C for 30 minutes and then cooled to room temperature. This was then concentrated at 50°C under reduced pressure, then firstly crystallised and secondly recrystallised from ethyl acetate/hexane to give **18** as dark purple fine needles, (4.18 g, 8.41 mmol, 58%). mp 109-110°C (ethyl acetate/hexane); (Found: C, 60.50; H, 5.09; N, 11.28. $C_{25}H_{25}ClN_4O_5$ requires C, 60.42; H, 5.07; N, 11.27%); ν_{max}(Nujol)/cm^{-1} 1737, 1602, 1512, 1339; δ_H(300 MHz; $CDCl_3$) 8.33 (1 H, d, *J* 2.5, ArC**H**), 8.09 (1 H, dd, *J* 9.0, 2.5, ArC**H**), 7.91 (2 H, d, J_{AB} 9.2, ArC**H**), 7.74 (1 H, d, *J* 9.0, ArC**H**), 7.19 (2 H, d, J_{AB} 8.8, ArC**H**), 6.84 (2 H, d, J_{AB} 8.8, ArC**H**), 6.78 (2 H, d, J_{AB} 9.2, ArC**H**), 4.16

(2 H, t, *J* 5.8, ArOC**H**$_2$CH$_2$), 3.83 (2 H, t, *J* 5.8, ArOCH$_2$C**H**$_2$), 3.67 (3 H, s, CO_2**Me**), 3.60 (2 H, q, *J* 7.1, C**H**$_2$Me), 3.56 (2 H, s, ArC**H**$_2$), 1.28 (3 H, t, *J* 7.1, CH_2**Me**); δ_C(75 MHz; $CDCl_3$) 172.7 (C), 158.0 (C), 153.4 (C), 152.1 (C), 147.5 (C), 144.7 (C), 134.3 (C), 130.8 (2 CH), 127.4 (CH), 127.1 (C), 126.4 (CH), 123.0 (CH), 118.4 (2 CH), 115.0 (2 CH), 111.9 (2 CH), 65.7 (CH_2), 52.4 (CH_3), 50.3 (CH_2), 46.7 (CH_2), 40.6 (CH_2), 12.7 (CH_3); *m/z* (EI) 496 (M^+), 498 (M^++2); (CI) 497 (MH^+), 499 (MH^++2); COSY and HMQC spectra both exhibited a good correlation with the proposed structure.

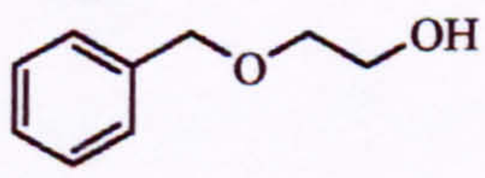
[4-(2-{[4-(2-Chloro-4-nitrophenylazo)-phenyl]-ethylamino}-ethoxy)-phenyl]-acetic acid, **24**.[58]



To a solution of the protected disperse red derivative **18** (1.08 g, 2.17 mmol) in tetrahydrofuran (150 ml) and water (10 ml) was added lithium hydroxide monohydrate (182 mg, 4.34 mmol 2.0 eq). The reaction mixture was stirred at 45°C for 30 minutes after which time TLC (ethyl acetate:hexane, 4:1) indicated that the starting material had been consumed. This was then cooled to room temperature, concentrated at 30°C under reduced pressure, diluted with dichloromethane (40 ml), washed with HCl (2 M, 3 x 30 ml), water (3 x 30 ml),

dried ($MgSO_4$) and concentrated in *vacuo* to give **24** as a red powder, (828 mg, 1.71 mmol, 79%). mp 192-193°C (ethyl acetate/hexane); (Found: C, 59.43; H, 4.82; N, 11.65. $C_{24}H_{23}ClN_4O_5$ requires C, 59.69; H, 4.80; N, 11.60%); ν_{max}(Nujol)/cm^{-1} 1695, 1601, 1511, 1337; δ_H(300 MHz; $CDCl_3$) 12.26 (1 H, br s, CO_2**H**), 8.37 (1 H, d, *J* 2.5, ArC**H**), 8.20 (1 H, dd, *J* 9.0, 2.5, ArC**H**), 7.84 (2 H, d, J_{AB} 9.3, ArC**H**), 7.74 (1 H, d, *J* 9.0, ArC**H**), 7.17 (2 H, d, J_{AB} 8.7, ArC**H**), 6.94 (2 H, d, J_{AB} 9.3, ArC**H**), 6.88 (2 H, d, J_{AB} 8.7, ArC**H**), 4.17 (2 H, t, *J* 5.4, ArOC**H**$_2$$CH_2$), 3.86 (2 H, t, *J* 5.4, ArOCH_2C**H**$_2$), 3.62 (2 H, q, *J* 7.0, C**H**$_2$Me), 3.48 (2 H, s, ArC**H**$_2$), 1.19 (3 H, t, *J* 7.0, CH_2**Me**); δ_C(75 MHz; $CDCl_3$) 173.3 (C), 157.4 (C), 152.6 (C), 152.5 (C), 147.0 (C), 143.7 (C), 132.7 (C), 130.8 (2 CH), 127.7 (C), 127.1 (CH), 126.0 (CH), 123.6 (CH), 118.3 (2 CH), 114.5 (2 CH), 112.2 (2 CH), 65.7 (CH_2), 49.6 (CH_2), 45.8 (CH_2), 40.1 (CH_2), 12.4 (CH_3); *m/z* (EI) 482 (M^+), 484 (M^++2); (CI) 483 (MH^+), 485 (MH^++2); COSY and HMQC spectra both exhibited a good correlation with the proposed structure.

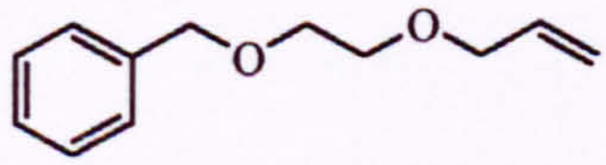
2-Benzyloxyethanol, **25**.[95]



Ethylene glycol (80 ml, 1.44 mol, 8 eq) was heated to 90°C and sodium hydroxide (7.2 g, 180 mmol, 1 eq) and 3 A molecular sieves (60 g) were added. The reaction mixture was stirred at 90°C until the sodium hydroxide dissolved (ca. 3 hours), after which time benzylbromide (21.8 ml, 180 mmol, 1 eq) was carefully added and the reaction mixture was stirred for a further 3 hours at 90°C.

The reaction mixture was then cooled to room temperature, diluted with acetone and the molecular sieves removed by filtration. The filtrate was concentrated under reduced pressure, diluted with ethyl acetate (60 ml) and washed with water (3 x 40 ml). The organic extract was dried ($MgSO_4$) and concentrated at 40°C under reduced pressure and the product eluted by flash column chromatography (ethyl acetate:hexane, 1:4) to give **25** as a clear colourless oil, (16.6 g, 109 mmol, 61%). ν_{max}(Nujol)/cm^{-1} 3403; δ_H(300 MHz; $CDCl_3$) 7.34-7.22 (5 H, m, ArC**H**) 4.50 (2 H, s, ArC$\mathbf{H}_2$), 3.68 (2 H, t, *J* 4.7, $CH_2C\mathbf{H}_2OH$), 3.51 (2 H, t, *J* 4.7, $C\mathbf{H}_2CH_2OH$), 3.08 (1 H, s, O**H**); δ_C(75 MHz; $CDCl_3$) 138.4 (C), 129.4 (CH), 128.9 (CH), 128.3 (CH), 128.2 (CH), 127.8 (CH), 73.6 (CH_2), 71.7 (CH_2), 62.1 (CH_2); *m/z* (EI) 152 (M^+); (CI) 153 (MH^+), 170 (MNH_4^+) (Found: MNH_4^+, 170.1181. $C_9H_{12}O_2$ requires MNH_4^+, 170.1179).

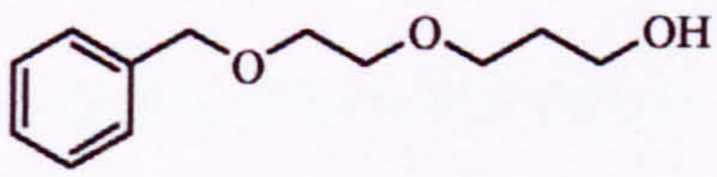
(2-Allyloxyethoxymethyl)-benzene, **26**.[96]



To a solution of mono protected ethylene glycol **25** (10.0 g, 65.8 mmol) dissolved in tetrahydrofuran (50 ml) was carefully added sodium hydride [50% w/w dispersion in mineral oil], (3.47 g, 72 mmol, 1.1 eq) and allyl bromide (6.7 ml, 79 mmol, 1.2 eq) at 0°C. The reaction mixture was stirred at room temperature for 4 hours after which time TLC (ethyl acetate:hexane, 1:4)

indicated that the starting material had been consumed. This was carefully quenched with water at 0°C, diluted with diethyl ether (40 ml), washed with water (3 x 30 ml), dried ($MgSO_4$), and concentrated in *vacuo*. The product was eluted by flash column chromatography (ethyl acetate:hexane, 1:49) to give **26** as a clear colourless oil, (9.79 g, 51.0 mmol, 78%). (Found: C, 74.80; H, 8.37. $C_{12}H_{16}O_2$ requires C, 74.97; H, 8.39%); ν_{max}(Nujol)/cm^{-1} 1645; δ_H(300 MHz; $CDCl_3$) 7.38-7.22 (5 H, m, ArC**H**), 5.93 (1 H, ddt, *J* 17.3, 10.4, 5.7, $CH_2C\mathbf{H}CH_2O$), 5.28 (1 H, ddt, *J* 17.3, 1.9, 1.3, *trans*-$C\mathbf{H}_2CHCH_2O$), 5.18 (1 H, ddt, *J* 10.4, 1.9, 1.3, *cis*-$C\mathbf{H}_2CHCH_2O$), 4.56 (2 H, s, Ar$C\mathbf{H}_2$), 4.02 (2 H, dt, *J* 5.7, 1.3, $CH_2CHC\mathbf{H}_2O$), 3.62 (4 H, br s, $OC\mathbf{H}_2C\mathbf{H}_2$); δ_C(75 MHz; $CDCl_3$) 138.7 (C), 135.2 (CH), 128.8 (2 CH), 128.2 (2 CH), 128.0 (CH), 117.5 (CH_2), 73.7 (CH_2), 72.7 (CH_2), 69.9 (CH_2), 69.8 (CH_2); *m/z* (EI) 192 (M^+); (CI) 210 (MNH_4^+). COSY spectra exhibited a good correlation with the proposed structure.

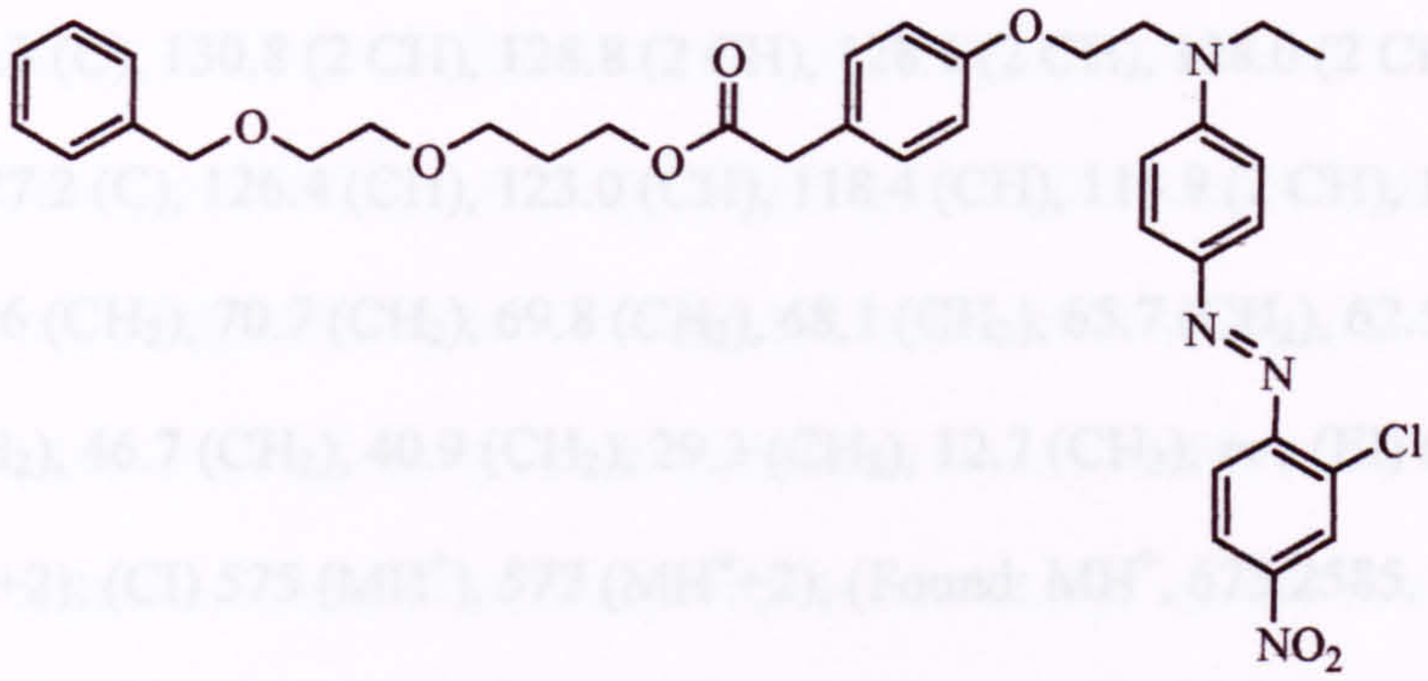
3-(2-Benzyloxyethoxy)-propan-1-ol, **27**.[97]



To a solution of the allyl substrate **26** (4 g, 20.8 mmol) in toluene (30 ml) was added borane methyl sulfide (2 M in toluene, 10.4 ml, 20.8 mmol) at 0°C. The reaction mixture was stirred at room temperature for 3 hours after which time

TLC (ethyl acetate:hexane,1:4) indicated that the starting material had been consumed. This was then quenched carefully at 0°C with methanol (37 ml), then hydrogen peroxide (30% aq. 2.8 ml, 25 mmol, 1.2 eq) and sodium hydroxide (2 M, 26 ml, 52 mmol, 2.5 eq) were carefully added. The reaction mixture was stirred at room temperature for 2 hours. This was then washed with water (3 x 40 ml), dried ($MgSO_4$) and concentrated in *vacuo*. The product was eluted by flash column chromatography (ethyl acetate:hexane, 1:9) to give **27** as a clear very pale yellow oil, (1.97 g, 9.38 mmol, 45%). ν_{max}(Nujol)/cm^{-1} 3417; δ_H(300 MHz; $CDCl_3$) 7.39-7.23 (5 H, m, ArC**H**), 4.53(2 H, s, ArC$\mathbf{H}_2$), 3.69 (2 H, t, *J* 6.0, $CH_2CH_2C\mathbf{H}_2OH$), 3.60 (2 H, t, *J* 6.0, $C\mathbf{H}_2CH_2CH_2OH$), 3.59 (4 H, br s, $OC\mathbf{H}_2C\mathbf{H}_2$), 3.31 (1 H, s, O**H**), 1.80 (2 H, quint, *J* 6.0, $CH_2C\mathbf{H}CH_2O$); δ_C(75 MHz; $CDCl_3$) 138.5 (C), 130.4 (2 CH), 129.3 (2 CH), 128.0 (CH), 73.6 (CH_2), 70.6 (CH_2), 70.0 (CH_2), 69.7 (CH_2), 61.2 (CH_2), 32.8 (CH_2); *m/z* (EI) 210 (M^+); (CI) 211 (MH^+); *m/z* (Found: MNH_4^+, 228.1599. $C_{12}H_{18}O_3$ requires MNH_4^+, 228.1600). COSY spectra exhibited a good correlation with the proposed structure.

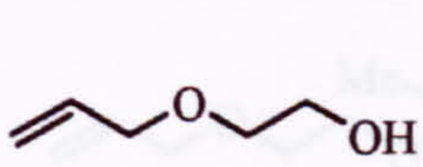
[4-(2-{[4-(2-Chloro-4-nitrophenylazo)-phenyl]-ethylamino}-ethoxy)-phenyl]-acetic acid 3-(2-benzyloxyethoxy)-propyl ester, **29**.[59, 98]



To a suspension of **24** (500 mg, 1.04 mmol) in toluene (60 ml) oxalyl chloride (0.10 ml, 1.14 mmol, 1.1 eq) and one drop of dimethylformamide were added and the reaction mixture was stirred at 40°C for 1 hour. This was then concentrated at 50°C under reduced pressure, dissolved in dichloromethane (30 ml) and quenched with triethylamine (10 ml). The alcohol **27** (326 mg, 1.55 mmol, 1.5 eq) was then added and the reaction mixture stirred at room temperature for 2 hours after which time TLC (ethyl acetate:hexane, 2:3) indicated that the starting material **24** had been consumed. The reaction mixture was washed with HCl (2 M, 3 x 30 ml), water (3 x 30 ml), dried ($MgSO_4$), and concentrated in *vacuo*. The product was eluted by flash column chromatography (ethyl acetate:hexane, 1:4) to give **29** as a dark metallic green oil, (216 mg, 0.32 mmol, 31%). ν_{max}(Nujol)/cm^{-1} 1736, 1598, 1518, 1337; δ_H(300 MHz; $CDCl_3$) 8.35 (1 H, d, *J* 2.4, ArC**H**), 8.12 (1 H, dd, *J* 8.9, 2.4, ArC**H**), 7.93 (2 H, d, J_{AB} 9.2, ArC**H**), 7.76 (1 H, d, *J* 8.9, ArC**H**), 7.35-7.23 (5 H, m, ArC**H**), 7.18 (2 H, d, J_{AB} 8.8, ArC**H**), 6.83 (2 H, d, J_{AB} 8.8, ArC**H**), 6.80 (2 H, d, J_{AB} 9.2, ArC**H**), 4.55 (2 H, s, ArC$\mathbf{H}_2$), 4.18 (2 H, t, *J* 6.4, $CH_2CH_2C\mathbf{H}_2O_2C$), 4.16 (2 H, t, *J* 5.5,

BnOC**H**$_2$CH$_2$), 3.84 (2 H, t, *J* 5.5, BnOCH$_2$C**H**$_2$), 3.64-3.48 (10 H, m, C**H**$_2$), 1.90 (2 H, quint, *J* 6.4, CH$_2$C**H**$_2$CH$_2$O$_2$C), 1.28 (3 H, t, *J* 7.1, CH$_2$**Me**); δ_C(75 MHz; $CDCl_3$) 172.2 (C), 157.9 (C), 153.5 (C), 152.1 (C), 147.5 (C), 144.8 (C), 138.6 (C), 134.3 (C), 130.8 (2 CH), 128.8 (2 CH), 128.1 (2 CH), 128.0 (2 CH), 127.4 (CH), 127.2 (C), 126.4 (CH), 123.0 (CH), 118.4 (CH), 114.9 (2 CH), 111.9 (2 CH), 73.6 (CH_2), 70.7 (CH_2), 69.8 (CH_2), 68.1 (CH_2), 65.7 (CH_2), 62.5 (CH_2), 50.4 (CH_2), 46.7 (CH_2), 40.9 (CH_2), 29.3 (CH_2), 12.7 (CH_3); *m/z* (EI) 674 (M^+), 676 (M^++2); (CI) 575 (MH^+), 577 (MH^++2); (Found: MH^+, 675.2585. $C_{36}H_{39}{}^{35}ClN_4O_7$ requires MH^+, 675.2585). COSY and HMQC spectra both exhibited a good correlation with the proposed structure.

2-Allyloxyethanol, **30**.

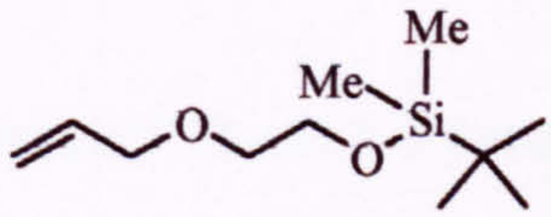


Ethylene glycol (150 ml, 2.7 mol, 5 eq) was heated to 90°C and sodium hydroxide (21.6 g, 540 mmol, 1 eq) and 3 A molecular sieves (100 g) were added at 60°C. The reaction mixture was stirred at 90°C until the sodium hydroxide dissolved (ca. 3 hours), after which time allyl bromide (45.7 ml, 540 mmol, 1 eq) was carefully added. The reaction mixture was stirred at 60°C for 6 hours. The reaction mixture was then cooled to room temperature, diluted with acetone and the molecular sieves removed by filtration. The filtrate was concentrated at 40°C under reduced pressure, diluted with ethyl acetate (50 ml) and washed with water (3 x 40 ml). The organic extract was dried ($MgSO_4$) and concentrated in *vacuo*. The product was eluted by flash column chromatography (ethyl acetate:hexane,

1:9) to give **30** as a clear colourless oil, (21.5 g, 211 mmol, 39%).

ν_{max}(Nujol)/cm^{-1} 3406 (O**H**); δ_H(300 MHz; $CDCl_3$) 5.93 (1 H, ddt, *J* 17.3, 10.4, 5.7, $CH_2C\mathbf{H}CH_2O$) 5.29 (1 H, ddd, *J* 17.3, 3.0, 1.5, *trans*-$C\mathbf{H}_2CHCH_2O$), 5.21 (1 H, ddd, *J* 10.4, 3.0, 1.5, *cis*-$C\mathbf{H}_2CHCH_2O$), 4.04 (2 H, dt, *J* 5.7, 1.5, $CH_2CHC\mathbf{H}_2O$), 3.75 (2 H, t, *J* 4.6, $HOC\mathbf{H}_2CH_2$) 3.56 (2 H, t, *J* 4.6, $HOCH_2C\mathbf{H}_2$), 2.41 (1 H, s, O**H**); δ_C(75 MHz; $CDCl_3$) 134.2 (CH), 117.0 (CH_2), 71.8 (CH_2), 71.2 (CH_2), 61.3 (CH_2); *m/z* (CI) 120 (MNH_4^+) (Found: MNH_4^+, 120.1026. $C_5H_{10}O_2$ requires MNH_4^+, 120.1025). COSY and HMQC spectra both exhibited a good correlation with the proposed structure.

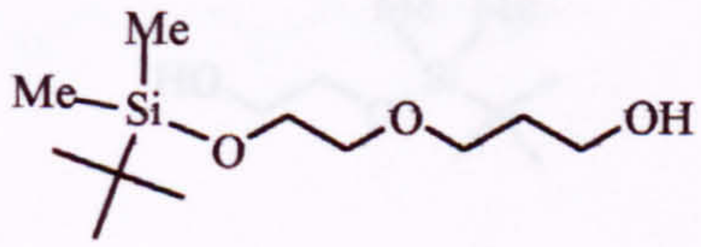
(2-Allyloxyethoxy)-tert-butyldimethylsilane, **31**.[61, 62]



To a solution of the allyl substrate **30** (3 g, 29 mmol) and imidazole (3 g, 44 mmol, 1.5 eq) in dichloromethane (20 ml), *t*-butyldimethyl silane (3 g, 44 mmol, 1.5 eq) were added at 0°C. The reaction mixture was stirred at room temperature for 3 hours after which time TLC (ethyl acetate:hexane, 1:4) indicated that the starting material had been consumed. This was then washed with water (3 x 30 ml), dried ($MgSO_4$) and concentrated in *vacuo*. The product was eluted by flash column chromatography (ethyl acetate:hexane, 1:99) to give **31** as a clear colourless oil, (5.0 g, 23 mmol, 80%). (Found: C, 61.28; H, 11.15. $C_{11}H_{24}O_2Si$ requires C, 61.05; H, 11.18%); ν_{max}(Nujol)/cm^{-1} 3374; δ_H(300 MHz; $CDCl_3$) 5.90

(1 H, ddt, *J* 17.3, 10.6, 5.6, CH_2CHCH_2O) 5.27 (1 H, ddt, *J* 17.3, 1.7, 1.7, *trans*-CH_2CHCH_2O), 5.16 (1 H, ddt, *J* 10.6, 1.5, 1.3, *cis*-CH_2CHCH_2O), 4.02 (2 H, dt, *J* 5.6, 1.4, CH_2CHCH_2O), 3.77 (2 H, t, *J* 5.4, OCH_2CH_2) 3.51 (2 H, t, *J* 5.4, OCH_2CH_2), 0.90 (9 H, s, $SiCMe_3$), 0.09 (6 H, s, $SiMe_2$); δ_C(75 MHz; $CDCl_3$) 135.9 (CH), 117.7 (CH_2), 73.2 (CH_2), 72.6 (CH_2), 63.8 (CH_2), 26.9 (CH_3), 19.4 (C), -4.3 (CH_3); *m/z* (CI) 217 (MH^+); COSY and HMQC spectra both exhibited a good correlation with the proposed structure.

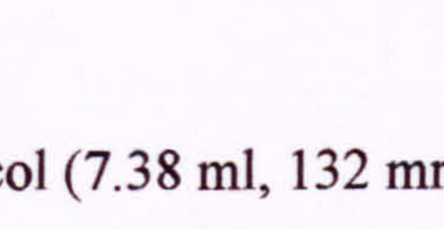
3-[2-(tert-Butyldimethylsilanyloxy)-ethoxy]-propan-1-ol, **33**.[48]



To a solution of the allyl substrate **31** (2.5 g, 11.6 mmol) in toluene (10 ml) was added borane dimethyl sulfide (1.1 ml, 11.6 mmol) at 0°C. The reaction mixture was stirred at room temperature for 30 minutes after which time TLC (ethyl acetate:hexane, 1:4) indicated that the starting material had been consumed. This was then quenched carefully at 0°C with methanol (4 ml), then hydrogen peroxide (30% aq. 2.6 ml, 23 mmol, 2 eq) and sodium hydroxide (920 mg, 23 mmol, 2 eq) was carefully added. The reaction mixture was stirred at room temperature for 8 hours. This was then washed with water (3 x 30 ml), dried ($MgSO_4$) and concentrated in *vacuo*. The product was eluted by flash column chromatography (ethyl acetate:hexane, 1:49) to give **33** as a clear colourless oil,

(1.1 g, 4.7 mmol, 41%). ν_{max}(Nujol)/cm^{-1} 3414; δ_H(300 MHz; $CDCl_3$) 3.75 (2 H, t, *J* 5.6, $CH_2CH_2C\mathbf{H}_2OH$), 3.74 (2 H, t, *J* 5.5, $TBSOC\mathbf{H}_2CH_2$), 3.66 (2 H, t, *J* 5.5, $TBSOCH_2C\mathbf{H}_2$), 3.51 (2 H, t, *J* 5.6, $C\mathbf{H}_2CH_2CH_2OH$), 2.68 (1 H, s, O**H**), 1.81 (2 H, quint, *J* 5.6, $CH_2C\mathbf{H}_2CH_2OH$), 0.88 (9 H, s, $SiC\mathbf{Me}_3$), 0.05 (6 H, s, $Si\mathbf{Me}_2$); δ_C(75 MHz; $CDCl_3$) 72.8 (CH_2), 71.0 (CH_2), 63.0 (CH_2), 62.1 (CH_2), 32.3 (CH_2), 26.1 (CH_3), 18.7 (C), -5.0 (CH_3); *m/z* (CI) 235 (MH^+), 252 (MNH_4^+) (Found: MNH_4^+, 252.1998. $C_{11}H_{26}O_3Si$ requires MNH_4^+, 252.1995).

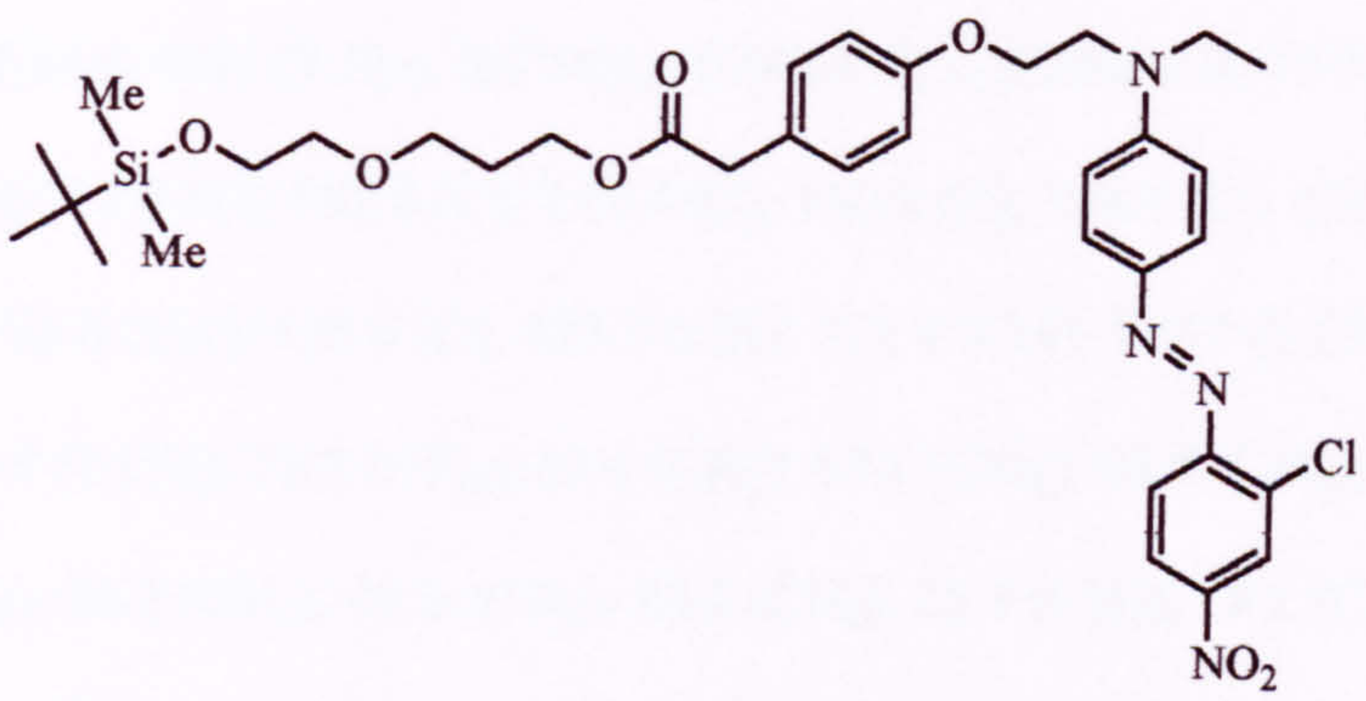
2-(tert-Butyldimethylsilanyloxy)-ethanol, **32**.[61, 62]



To a solution of ethylene glycol (7.38 ml, 132 mmol, 5 eq) and imidazole (3.6 g, 5.3 mmol, 2 eq) in dimethylformamide (100 ml), *tert*-butyldimethylsilyl chloride (4 g, 26.5 mmol) was carefully added at 0°C. The reaction mixture was stirred for 12 hours at room temperature after which TLC (ethyl acetate:hexane, 1:4, and PMA dip to visualise) indicated that the *tert*-butyldimethylsilyl chloride had been entirely consumed. The reaction mixture was diluted with water (70 ml) and the product extracted with diethyl ether (5 x 30 ml). The diethyl ether fractions were combined and washed with water (2 x 20 ml), dried ($MgSO_4$) and concentrated in *vacuo* to give a pale yellow oil. The product was eluted by flash column chromatography (ethyl acetate:hexane, 1:49) to give **32** as a clear colourless oil, (3.45 g, 19.6 mmol, 74%). (Found: C, 54.31; H, 11.34. $C_8H_{20}O_2Si$ requires C,

54.49; H, 11.43%); ν_{max}(Nujol)/cm^{-1} 3374; δ_H(300 MHz; $CDCl_3$) 3.69 (2 H, t, *J* 4.9, $CH_2C\mathbf{H}_2OH$), 3.61 (2 H, t, *J* 4.9, $C\mathbf{H}_2CH_2OH$), 2.42 (1 H, s, $CH_2CH_2O\mathbf{H}$), 0.87 (9 H, s, $SiC\mathbf{Me}_3$), 0.06 (6 H, s, $Si\mathbf{Me}_2$); δ_C(75 MHz; $CDCl_3$) 64.5 (CH_2), 64.1 (CH_2), 26.3 (CH_3), 18.7 (C), -5.0 (CH_3); *m/z* (CI) 177 (MH^+); COSY and HMQC spectra both exhibited a good correlation with the proposed structure.

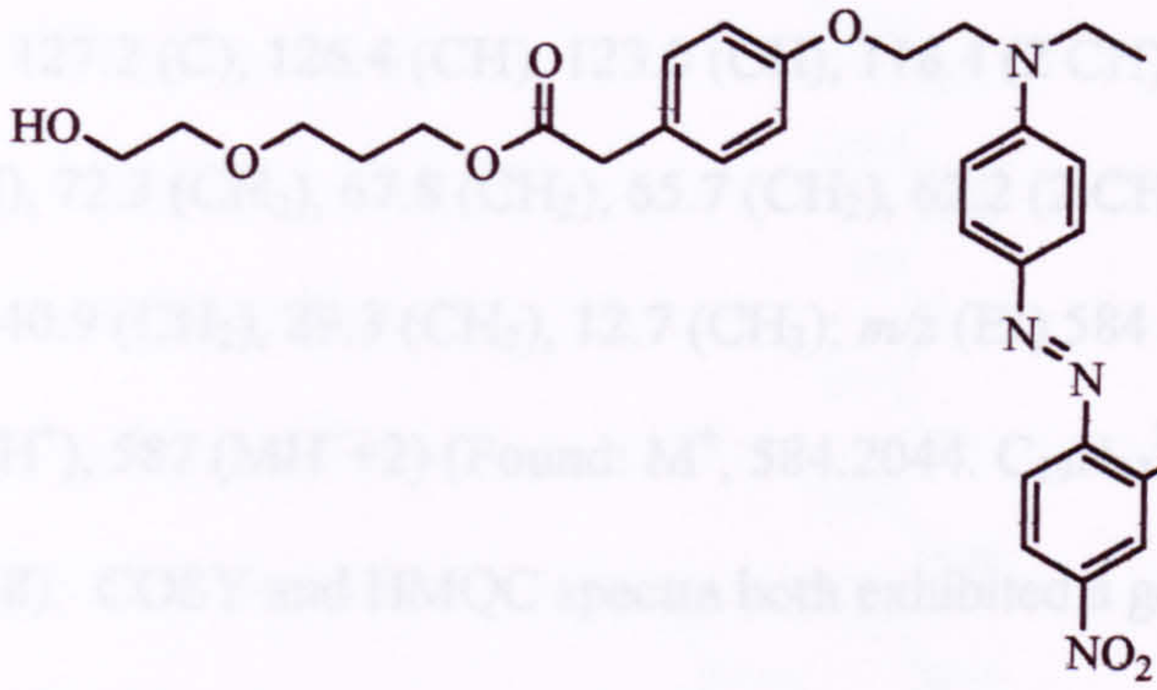
[4-(2-{[4-(2-Chloro-4-nitrophenylazo)-phenyl]-ethylamino}-ethoxy)-phenyl]-acetic acid 3-[2-(tert-butyldimethylsilanyloxy)-ethoxy]-propyl ester, **34**.[55-57]



To a solution of the alcohol **33** (2.57 g, 11.0 mmol), triphenylphosphine (2.90 g, 11.0 mmol) and diethyl azodicarboxylate (1.71 ml, 11.0 mmol) in toluene (300 ml) was added the acid **24** (3.55 g, 7.37 mmol, 0.67 eq). This was stirred at 50°C for 5 hours and then room temperature for 2 days after which time TLC (ethyl acetate:hexane, 2:3) indicated that the acid had been consumed. The solution was then concentrated at 50°C under reduced pressure, diluted with dichloromethane, washed with water (3 x 30 ml), dried ($MgSO_4$) and concentrated in *vacuo*. The product was eluted by flash column chromatography

(ethyl acetate:hexane, 1:4) to give **34** as a dark metallic green oil, (6.0 g, 8.60 mmol, 83%). mp 38-39°C (diethyl ether/cyclohexane); (Found: C, 60.07; H, 6.71; N, 7.99. $C_{35}H_{47}ClN_4O_7Si$ requires C, 60.11; H, 6.77; N, 8.01%); ν_{max}(Nujol)/cm^{-1} 1741, 1604, 1514, 1376; δ_H(300 MHz; $CDCl_3$) 8.39 (1 H, d, *J* 2.5, ArC**H**), 8.16 (1 H, dd, *J* 9.0, 2.5, ArC**H**), 7.96 (2 H, d, J_{AB} 8.9, ArC**H**), 7.79 (1 H, d, *J* 9.0, ArC**H**), 7.19 (2 H, d, J_{AB} 8.9, ArC**H**), 6.84 (4 H, d, J_{AB} 8.9, ArC**H**), 4.18 (2 H, t, *J* 5.8, ArOC**H**$_2$CH$_2$), 4.17 (2 H, t, *J* 6.1, CH$_2$CH$_2$C**H**$_2$O$_2$C), 3.86 (2 H, t, *J* 5.8, ArOCH$_2$C**H**$_2$), 3.73 (2 H, t, *J* 5.3, SiOCH$_2$C**H**$_2$), 3.63 (2 H, q, *J* 7.1 C**H**$_2$Me) 3.55 (2 H, s, ArC**H**$_2$) 3.49 (2 H, t, *J* 6.1, C**H**$_2$CH$_2$CH$_2$O$_2$C) 3.46 (2 H, t, *J* 5.3, SiOC**H**$_2$CH$_2$), 1.88 (2 H, quint, *J* 6.1, CH$_2$C**H**$_2$CH$_2$O$_2$C), 1.30 (2 H, t, *J* 7.1, CH$_2$**Me**), 0.89 (9 H, s, SiC**Me**$_3$), 0.06 (6 H, s, Si**Me**$_2$); δ_C(75 MHz; $CDCl_3$) 171.6 (C), 157.3 (C), 152.8 (C), 151.5 (C), 146.8 (C), 144.1 (C), 133.7 (C), 130.2 (2 CH), 126.8 (CH), 126.6 (C), 125.7 (CH), 122.4 (CH), 117.7 (2 CH), 114.2 (2 CH), 111.3 (2 CH), 72.2 (CH$_2$), 67.4 (CH$_2$), 65.1 (CH$_2$), 62.5 (CH$_2$), 61.9 (CH$_2$), 49.7 (CH$_2$), 46.1 (CH$_2$), 40.2 (CH$_2$), 28.8 (CH$_2$), 25.7 (CH$_3$), 18.2 (C), 12.1 (CH$_3$), -5.0 (CH$_3$); *m/z* (EI) 698 (M$^+$), 670 (M$^+$+2); (CI) 699 (MH$^+$), 701 (MH$^+$+2); COSY and HMQC spectra both exhibited a good correlation with the proposed structure.

[4-(2-{[4-(2-Chloro-4-nitrophenylazo)-phenyl]-ethylamino}-ethoxy)-phenyl]-acetic acid 3-(2-hydroxyethoxy)-propyl ester, **14**.[61]



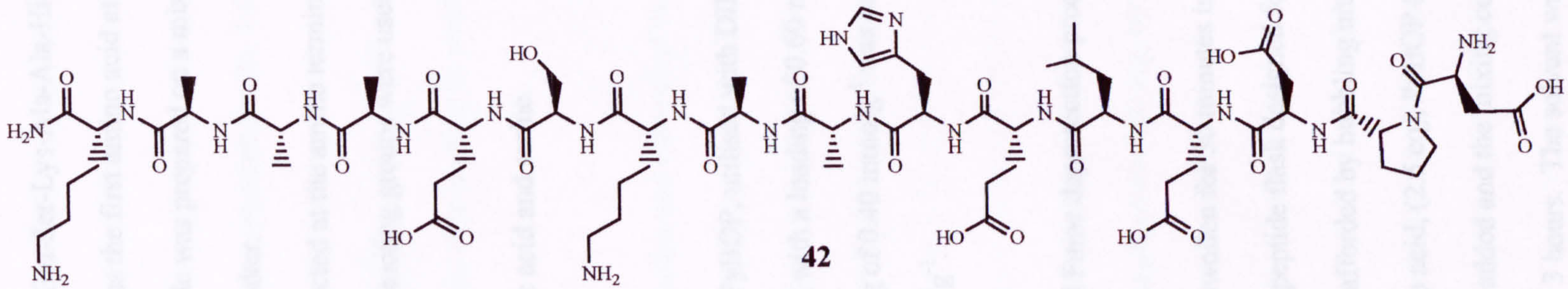
To a solution of **34** (70 mg, 0.10 mmol) in tetrahydrofuran (5 ml) was added tetrabutylammonium fluoride trihydrate (35 mg, 0.11 mmol, 1.1 eq). The reaction mixture was stirred at 40°C for 2 hours after which time TLC (triethylamine:ethyl acetate:hexane, 1:20:30) indicated that the starting material had been consumed. This was then diluted with diethyl ether (30 ml), washed with 1 M HCl (3 x 30 ml), water (3 x 30 ml), dried ($MgSO_4$), and concentrated in *vacuo*. The product was eluted by flash column chromatography (ethyl acetate:hexane, 1:4) to give **14** as a dark metallic green oil, (20 mg, 34 μmol, 34%). mp 92-93°C (ethyl acetate/hexane); ν_{max}(Nujol)/cm^{-1} 1714, 1603, 1509, 1462, 1377; δ_H(300 MHz; $CDCl_3$) 8.38 (1 H, d, *J* 2.4, ArC**H**), 8.14 (1 H, dd, *J* 9.0, 2.4, ArC**H**), 7.95 (2 H, d, J_{AB} 8.9, ArC**H**), 7.78 (1 H, d, *J* 9.0, ArC**H**), 7.19 (2 H, d, J_{AB} 8.7, ArC**H**), 6.85 (2 H, d, J_{AB} 8.7, ArC**H**), 6.82 (2 H, d, J_{AB} 8.9, ArC**H**), 4.20 (2 H, t, *J* 6.3, $CH_2CH_2C\mathbf{H}_2O_2C$), 4.18 (2 H, t, *J* 5.8, $ArOC\mathbf{H}_2CH_2$), 3.86 (2 H, t, *J* 5.8, $ArOCH_2C\mathbf{H}_2$), 3.69 (2 H, t, *J* 4.5, $HOC\mathbf{H}_2CH_2$), 3.62 (2 H, q, *J* 7.1, $C\mathbf{H}_2Me$) 3.51 (2 H, s, $ArC\mathbf{H}_2$) 3.49 (2 H, t, *J* 4.5, $HOCH_2C\mathbf{H}_2$) 3.48 (2 H, t,

J 6.3, C**H**$_2$CH$_2$CH$_2$O$_2$C), 2.07 (1 H, s, **H**OCH$_2$CH$_2$), 1.89 (2 H, quint, *J* 6.3, CH$_2$C**H**$_2$CH$_2$O$_2$C), 1.29 (3 H, t, *J* 7.1, CH$_2$**Me**); δ_C(75 MHz; $CDCl_3$) 171.6 (C), 157.3 (C), 152.8 (C), 151.5 (C), 146.8 (C), 144.1 (C), 133.7 (C), 130.8 (2 CH), 127.4 (CH), 127.2 (C), 126.4 (CH), 123.0 (CH), 118.4 (2 CH), 114.9 (2 CH), 112.0 (2 CH), 72.3 (CH_2), 67.8 (CH_2), 65.7 (CH_2), 62.2 (2 CH_2), 50.4 (CH_2), 46.7 (CH_2), 40.9 (CH_2), 29.3 (CH_2), 12.7 (CH_3); *m/z* (EI) 584 (M^+), 586 (M^++2); (CI) 585 (MH^+), 587 (MH^++2) (Found: M^+, 584.2044. $C_{29}H_{33}{}^{35}ClN_4O_7$ requires M^+, 584.2038). COSY and HMQC spectra both exhibited a good correlation with the proposed structure.

Synthesis of the protected and deprotected16 residue peptide on Tentagel **40** *and* **41** *and the cleaved peptide* **42**.

Amino terminal from rink linker cleavage

Free amino end of the sequence



42

The 16 residue peptide was sequentially synthesised *via* a conventional manual solid phase Fmoc/TFA strategy on Tentagel polymer support. The peptide's sequence was HO-Lys-Ala-Ala-Ala-Glu-Ser-Lys-Ala-Ala-His-Glu-Leu-Glu-Asp-Pro-Asp-NH_2, where L-lysine was the first amino acid attached, and L-aspartic acid was the last. The peptide was prepared on a mixture of 90% Tentagel and 10% acid labile Rink linker.

The amino acids were all Fmoc protected at the amino terminal, leaving the acid terminal unprotected. Additional protecting groups were used for the side chains if necessary. These were;

a) *t*-Butyl for aspartic acid, glutamic acid and serine.

b) *t*-Butyl carbamate for lysine.

c) Trityl for histidine.

The coupling reagent of choice was PyBOP, utilised with DIPEA and HOBt in dimethylformamide. 2 g of Tentagel with a loading of 0.60 $mmolg^{-1}$ and 200 mg of Tentagel rink linker with a loading of 0.40 $mmolg^{-1}$ was used, giving a mixture with a loading of 0.58 $mmolg^{-1}$.

The general amino acid coupling and Fmoc deprotection procedures were;

a) The Tentagel resin (1.0 eq) was swollen for 30 minutes in dimethylformamide (15 ml) in a peptide flask designed for nitrogen bubbling and suction. Gentle mixing was afforded by bubbling nitrogen through the suspension. The protected amino acid, (2.5 eq), PyBOP (2.5 eq), HOBt (2.5 eq) and DIPEA (5 eq) were then added and the mixing continued by bubbling nitrogen through the solution for 3 hours. The solvent was then removed *via*

filtration and the support washed with dimethylformamide (2 x 20 ml), dichloromethane (2 x 20 ml), methanol (2 x 20 ml) and dimethylformamide (1 x 20 ml). The conversion was verified by a negative Kaiser and TNBS test. A double coupling was undertaken if necessary.

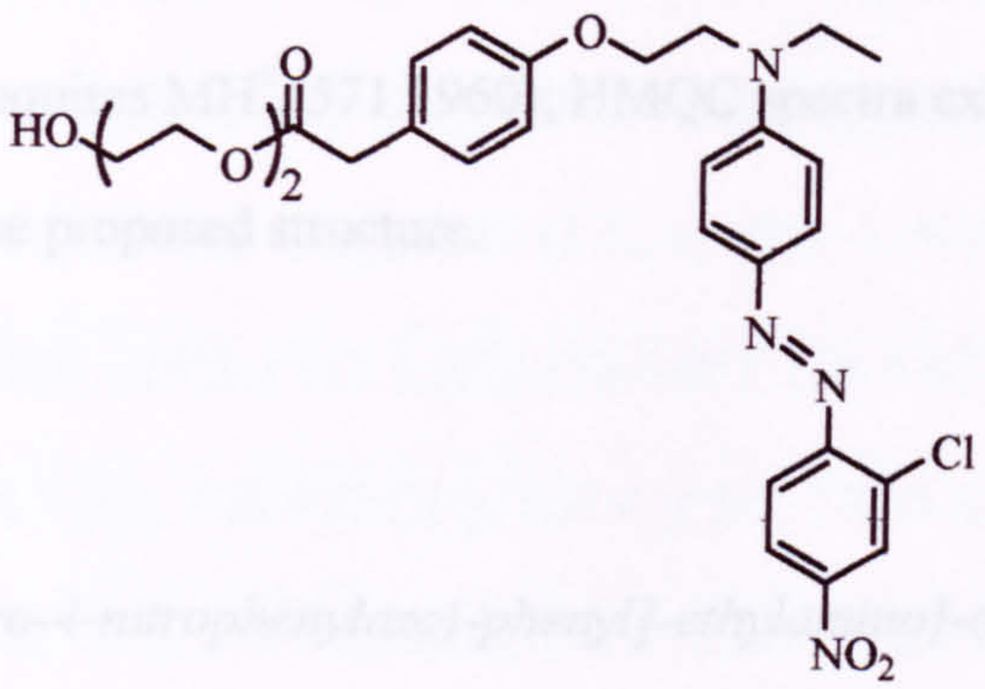
b) The resin functionalised with an Fmoc protected amino acid was gently mixed in a solution of piperidine:dimethylformamide, 1:4, by bubbling nitrogen through for 20 minutes. The solution was then removed by filtration and the resin washed with dimethylformamide (2 x 20 ml). The Kaiser and TNBS tests indicated the successful deprotection by yielding a positive result.

Upon coupling the last amino acid residue and removal of its Fmoc protection, the bulk resin was washed with dimethylformamide (2 x 20 ml), dichloromethane (2 x 20 ml) and methanol (2 x 20 ml) and dried under reduced pressure to give **40** as a pale yellow resin (3.8 g, 71%). The yield was based on the expected increase of the resin's weight.

Removal of the peptide's side chain protecting groups and partial cleavage of the peptide was performed on a small sample of **40** (200 mg) by gently bubbling nitrogen through a suspension of the resin in a solution of triisopropylamine:TFA, 1:49 (2 ml) for 1 hour. The solution was removed by filtration and the filtrate was collected and dried under reduced pressure to give an off white powder **42** (6 mg)); *m/z* (FAB) 1681 (M^+) (Found (ES) 561.2787 (MH_3^{3+}), 841.4056 (MH_2^{2+}), $C_{70}H_{114}N_{21}O_{27}$ requires (ES) 561.2809 (MH_3^{3+}), 841. 4175 (MH_2^{2+})).

The supported deprotected peptide was washed with dimethylformamide, dichloromethane and methanol, and dried under reduced pressure to give a pale yellow resin **41** (160 mg).

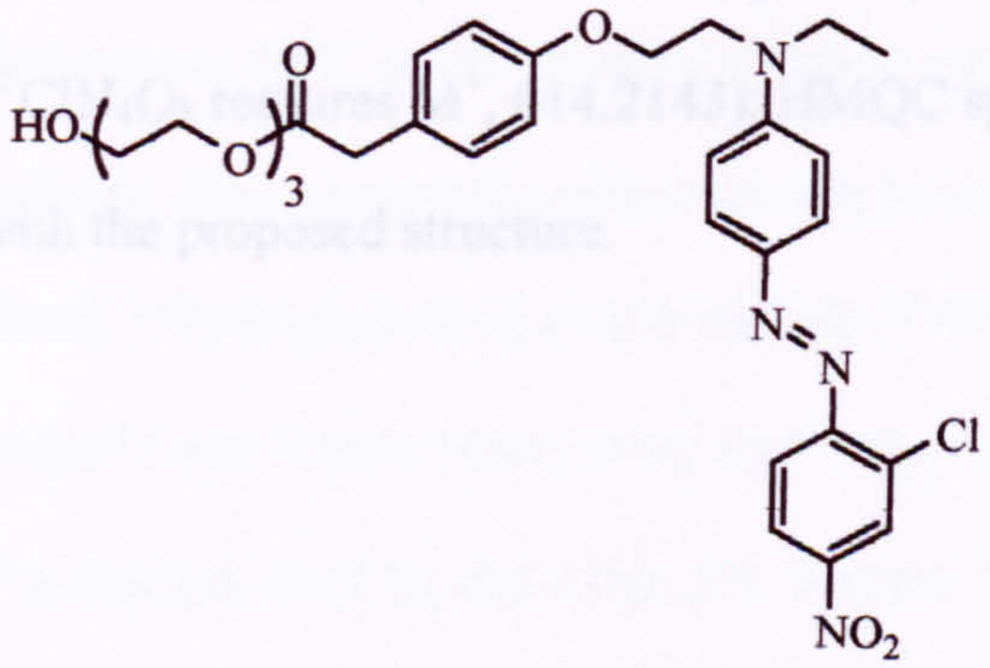
[4-(2-{[4-(2-Chloro-4-nitrophenylazo)-phenyl]-ethylamino}-ethoxy)-phenyl]-acetic acid-2-(2-hydroxyethoxy)-ethyl ester, **46**.[55-57]



To a solution of **24** (200 mg, 0.41 mmol), triphenylphosphine (162 mg, 0.62 mmol, 1.5 eq) and diethyl azodicarboxylate (108 mg, 0.62 mmol, 1.5 eq) in toluene (20 ml) diethylene glycol (66 mg, 0.62 mmol, 1.5 eq) was added. The reaction mixture was stirred for 5 days at room temperature after which TLC (methanol:ethyl acetate, 1:9) indicated that the acid had been entirely consumed. The toluene was removed at 50°C under reduced pressure and the product eluted by gradient flash column chromatography (ethyl acetate:hexane, 4:1; methanol:ethyl acetate, 1:9) to give **46** as a dark red oil, (190 mg, 0.33 mmol, 81%). ν_{max}(Nujol)/cm^{-1} 3451, 1731, 1599, 1514, 1335; δ_H(300 MHz; $CDCl_3$) 8.38 (1 H, d, *J* 2.4, ArC**H**), 8.14 (1 H, dd, *J* 9.0, 2.4, ArC**H**), 7.95 (2 H, d, J_{AB} 9.0, ArC**H**), 7.78 (1 H, d, *J* 9.0, ArC**H**), 7.21 (2 H, d, J_{AB} 9.3, ArC**H**), 6.85 (2 H, d, J_{AB} 9.0, ArC**H**), 6.82 (2 H, d, J_{AB} 9.3, ArC**H**), 4.26 (2 H, t, *J* 4.7, C$\mathbf{H}_2$), 4.18

(2 H, t, *J* 5.7, ArOC**H**$_2$CH$_2$), 3.86 (2 H, t, *J* 5.6, ArOCH$_2$C**H**$_2$), 3.72-3.67 (4 H, m, C**H**$_2$), 3.64-3.54 (6 H, m, C**H**$_2$), 1.95 (1 H, s, O**H**), 1.29 (3 H, t, *J* 7.0, CH$_2$**Me**); δ_C(75 MHz; $CDCl_3$) 172.2 (C), 158.0 (C), 153.5 (C), 152.0 (C), 147.5 (C), 144.8 (C), 134.3 (C), 130.8 (2 CH), 127.4 (CH), 127.0 (C), 126.4 (CH), 123.0 (CH), 118.4 (2 CH), 114.9 (2 CH), 112.0 (2 CH), 72.7 (CH_2), 69.4 (CH_2), 65.7 (CH_2), 64.2 (CH_2), 62.1 (CH_2), 50.4 (CH_2), 46.8 (CH_2), 40.7 (CH_2), 12.7 (CH_3); *m/z* (CI) 571 (MH^+), 573 (MH^++2) (Found: MH^+, 571.1947. $C_{28}H_{31}{}^{35}ClN_4O_7$ requires MH^+, 571.1960); HMQC spectra exhibited a good correlation with the proposed structure.

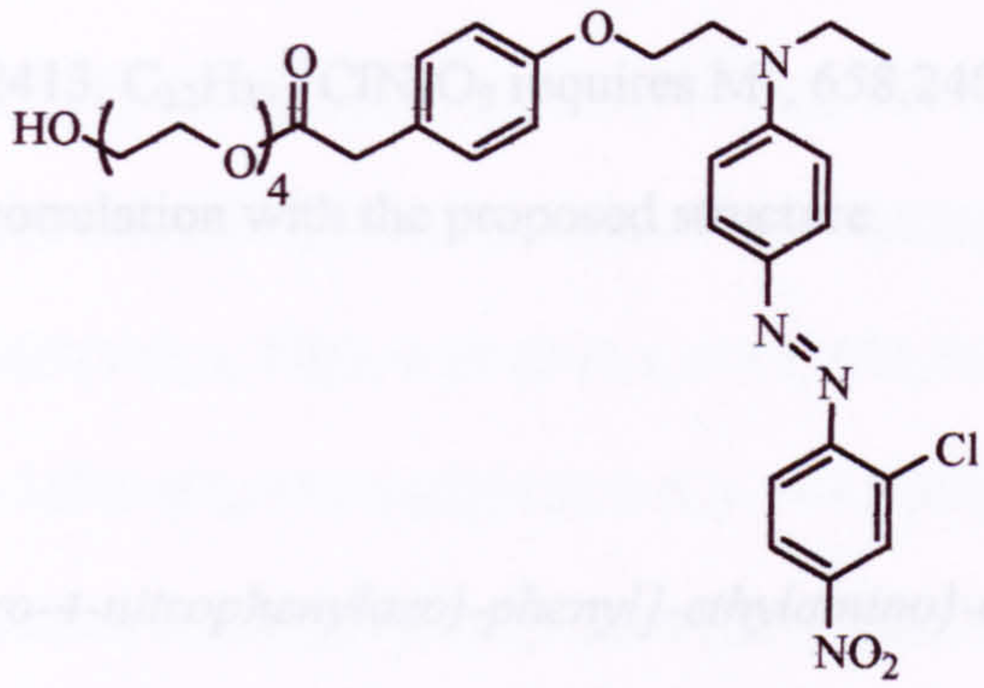
[4-(2-{[4-(2-Chloro-4-nitrophenylazo)-phenyl]-ethylamino}-ethoxy)-phenyl]-acetic acid 2-[2-(2-hydroxyethoxy)-ethoxy]-ethyl ester, **47**.[55-57]



To a solution of **24** (200 mg, 0.41 mmol), triphenylphosphine (162 mg, 0.62 mmol, 1.5 eq) and diethyl azodicarboxylate (108 mg, 0.62 mmol, 1.5 eq) in toluene (20 ml) was added triethylene glycol (93 mg, 0.62 mmol, 1.5 eq). The reaction mixture was stirred for 5 days at room temperature after which TLC (methanol:ethyl acetate, 1:9) indicated that the acid had been entirely consumed.

The toluene was removed at 50°C under reduced pressure and the product eluted by gradient flash column chromatography (ethyl acetate:hexane, 4:1; methanol:ethyl acetate, 1:9) to give **47** as a dark red oil, (180 mg, 0.29 mmol, 71%). ν_{max}(Nujol)/cm^{-1} 3439, 1736, 1599, 1513, 1336; δ_H(300 MHz; $CDCl_3$) 8.37 (1 H, d, *J* 2.5, ArC**H**), 8.13 (1 H, dd, *J* 9.0, 2.5, ArC**H**), 7.93 (2 H, d, J_{AB} 9.2, ArC**H**), 7.76 (1 H, d, *J* 9.0, ArC**H**), 7.20 (2 H, d, J_{AB} 8.7, ArC**H**), 6.84 (2 H, d, J_{AB} 8.7, ArC**H**), 6.81 (2 H, d, J_{AB} 9.2, ArC**H**), 4.25 (2 H, t, *J* 4.7, C**H**$_2$), 4.17 (2 H, t, *J* 5.7, ArOC**H**$_2$CH$_2$), 3.85 (2 H, t, *J* 5.7, ArOCH$_2$C**H**$_2$), 3.74-3.68 (4 H, m, C**H**$_2$), 3.66-3.58 (10 H, m, C**H**$_2$), 2.41 (1 H, s, O**H**), 1.29 (3 H, t, *J* 7.0, CH$_2$**Me**); δ_C(75 MHz; $CDCl_3$) 172.3 (C), 158.0 (C), 153.5 (C), 152.1 (C), 147.5 (C), 144.7 (C), 134.3 (C), 130.9 (2 CH), 127.4 (CH), 127.0 (C), 126.4 (CH), 123.0 (CH), 118.4 (2 CH), 114.9 (2 CH), 111.9 (2 CH), 72.9 (CH$_2$), 70.9 (CH$_2$), 70.7 (CH$_2$), 69.5 (CH$_2$), 65.7 (CH$_2$), 64.2 (CH$_2$), 62.1 (CH$_2$), 50.4 (CH$_2$), 46.7 (CH$_2$), 40.6 (CH$_2$), 12.7 (CH$_3$); *m/z* (CI) 615 (MH$^+$), 617 (MH$^+$+2) (Found: M$^+$, 614.2140. $C_{30}H_{35}{}^{35}ClN_4O_8$ requires M$^+$, 614.2143); HMQC spectra exhibited a good correlation with the proposed structure.

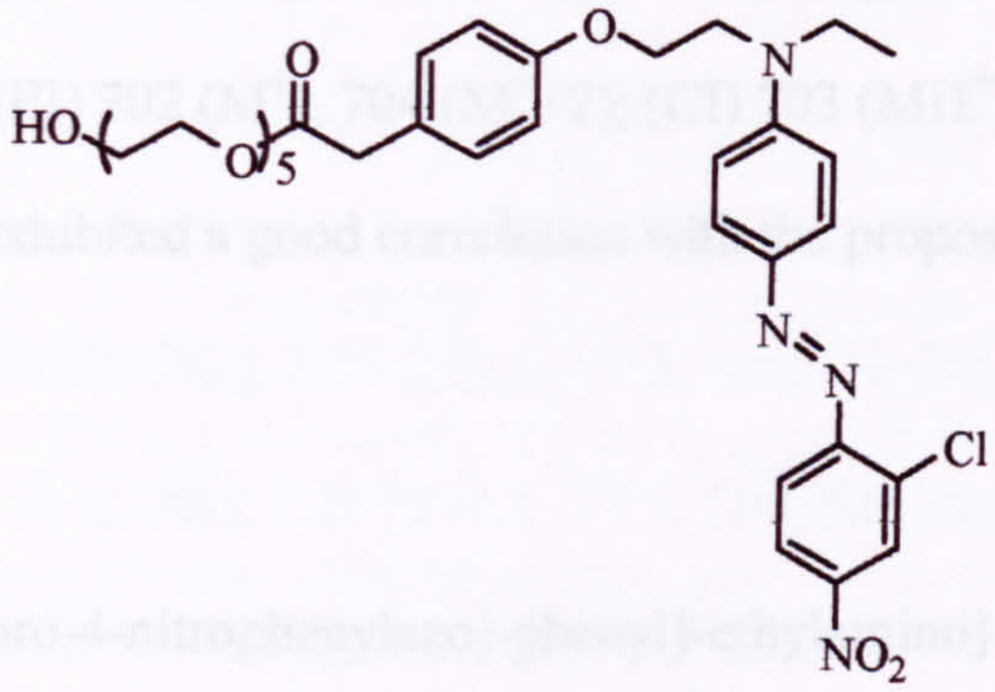
[4-(2-{[4-(2-Chloro-4-nitrophenylazo)-phenyl]-ethylamino}-ethoxy)-phenyl]-acetic acid 2-{2-[2-(2-hydroxyethoxy)-ethoxy]-ethoxy}-ethyl ester, **48**.[55-57]



To a solution of **24** (200 mg, 0.41 mmol), triphenylphosphine (162 mg, 0.62 mmol, 1.5 eq) and diethyl azodicarboxylate (108 mg, 0.62 mmol, 1.5 eq) in toluene (20 ml) was added tetraethylene glycol (120 mg, 0.62 mmol, 1.5 eq). The reaction mixture was stirred for 5 days at room temperature after which TLC (methanol:ethyl acetate, 1:9) indicated that the acid had been entirely consumed. The toluene was removed at 50°C under reduced pressure and the product eluted by gradient flash column chromatography (ethyl acetate:hexane, 4:1; methanol:ethyl acetate, 1:9) to give **48** as a dark red oil, (198 mg, 0.30 mmol, 73%). ν_{max}(Nujol)/cm^{-1} 3441, 1737, 1600, 1514, 1337; δ_H(300 MHz; $CDCl_3$) 8.37 (1 H, d, *J* 2.5, ArC**H**), 8.14 (1 H, dd, *J* 9.0, 2.5, ArC**H**), 7.94 (2 H, d, J_{AB} 9.1, ArC**H**), 7.77 (1 H, d, *J* 9.0, ArC**H**), 7.20 (2 H, d, J_{AB} 8.6, ArC**H**), 6.84 (2 H, d, J_{AB} 8.6, ArC**H**), 6.81 (2 H, d, J_{AB} 9.1, ArC**H**), 4.25 (2 H, t, *J* 4.8, C$\mathbf{H}_2$), 4.18 (2 H, t, *J* 5.7, ArOC$\mathbf{H}_2$CH$_2$), 3.86 (2 H, t, *J* 5.7, ArOCH$_2$C$\mathbf{H}_2$), 3.73-3.59 (18 H, m, C$\mathbf{H}_2$), 2.46 (1 H, s, O**H**), 1.29 (3 H, t, *J* 7.1, CH$_2$**Me**); δ_C(75 MHz; $CDCl_3$) 172.2 (C), 157.9 (C), 153.5 (C), 152.1 (C), 147.5 (C), 144.8 (C), 134.3 (C), 130.9 (2 CH), 127.4 (CH), 127.1 (C), 126.4 (CH), 123.0 (CH), 118.4 (2 CH), 114.9 (2

CH), 112.0 (2 CH), 72.9 (CH_2), 71.0 (CH_2), 70.9 (2 CH_2), 70.7 (CH_2), 69.5 (CH_2), 65.7 (CH_2), 64.3 (CH_2), 62.1 (CH_2), 50.4 (CH_2), 46.7 (CH_2), 40.6 (CH_2), 12.7 (CH_3); *m/z* (EI) 658 (M^+), 660 (M^++2); (CI) 659 (MH^+), 661 (MH^++2) (Found: M^+, 658.2413. $C_{32}H_{39}{}^{35}ClN_4O_9$ requires M^+, 658.2406); HMQC spectra exhibited a good correlation with the proposed structure.

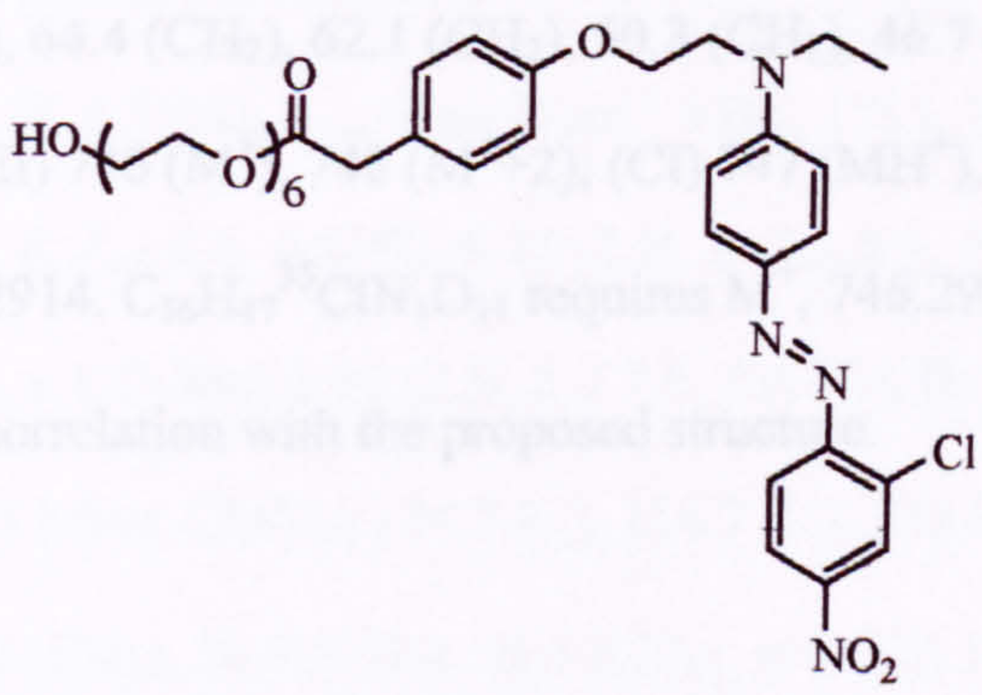
[4-(2-{[4-(2-Chloro-4-nitrophenylazo)-phenyl]-ethylamino}-ethoxy)-phenyl]-acetic acid 2-(2-{2-[2-(2-hydroxyethoxy)-ethoxy]-ethoxy}-ethoxy)-ethyl ester, **49**.



To a solution of **24** (200 mg, 0.41 mmol), triphenylphosphine (162 mg, 0.62 mmol, 1.5 eq) and diethyl azodicarboxylate (108 mg, 0.62 mmol, 1.5 eq) in toluene (20 ml) was added pentaethylene glycol (148 mg, 0.62 mmol, 1.5 eq). The reaction mixture was stirred for 5 days at room temperature after which TLC (methanol:ethyl acetate, 1:9) indicated that the acid had been entirely consumed. The toluene was removed at 50°C under reduced pressure and the product eluted by gradient flash column chromatography (ethyl acetate:hexane, 4:1; methanol:ethyl acetate, 1:9) to give **49** as a dark red oil, (225 mg, 0.32 mmol, 78%). (Found: C, 57.89; H, 6.17; N, 7.91. $C_{34}H_{43}ClN_4O_{10}$ requires C, 58.07; H,

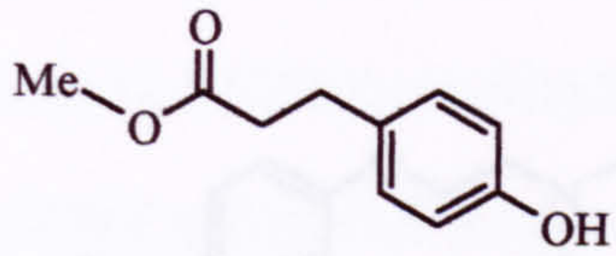
6.16; N, 7.97%); ν_{max}(Nujol)/cm^{-1} 3466, 1731, 1599, 1518, 1336; δ_H(300 MHz; $CDCl_3$) 8.37 (1 H, d, *J* 2.5, ArC**H**), 8.13 (1 H, dd, *J* 9.0, 2.5, ArC**H**), 7.94 (2 H, d, J_{AB} 9.3, ArC**H**), 7.77 (1 H, d, *J* 9.0, ArC**H**), 7.21 (2 H, d, J_{AB} 8.8, ArC**H**), 6.84 (2 H, d, J_{AB} 8.8, ArC**H**), 6.81 (2 H, d, J_{AB} 9.3, ArC**H**), 4.24 (2 H, t, *J* 4.8, C**H**$_2$), 4.17 (2 H, t, *J* 5.8, ArOC**H**$_2$CH$_2$), 3.85 (2 H, t, *J* 5.8, ArOCH$_2$C**H**$_2$), 3.73-3.58 (22 H, m, C**H**$_2$), 2.66 (1 H, s, O**H**), 1.29 (3 H, t, *J* 7.1, CH$_2$**Me**); δ_C(75 MHz; $CDCl_3$) 172.2 (C), 157.9 (C), 153.5 (C), 152.1 (C), 147.5 (C), 144.8 (C), 134.3 (C), 130.9 (2 CH), 127.4 (CH), 127.1 (C), 126.4 (CH), 123.0 (CH), 118.4 (2 CH), 114.9 (2 CH), 112.0 (2 CH), 72.9 (CH_2), 70.9 (5CH_2), 70.7 (CH_2), 69.4 (CH_2), 65.7 (CH_2), 64.3 (CH_2), 62.1 (CH_2), 50.4 (CH_2), 46.7 (CH_2), 40.6 (CH_2), 12.7 (CH_3); *m/z* (EI) 702 (M^+), 704 (M^++2); (CI) 703 (MH^+), 705 (MH^++2); HMQC spectra exhibited a good correlation with the proposed structure.

[4-(2-{[4-(2-Chloro-4-nitrophenylazo)-phenyl]-ethylamino}-ethoxy)-phenyl]-acetic acid 2-[2-(2-{2-[2-(2-hydroxyethoxy)-ethoxy]-ethoxy}-ethoxy)-ethoxy]-ethyl ester, **50**.[55-57]



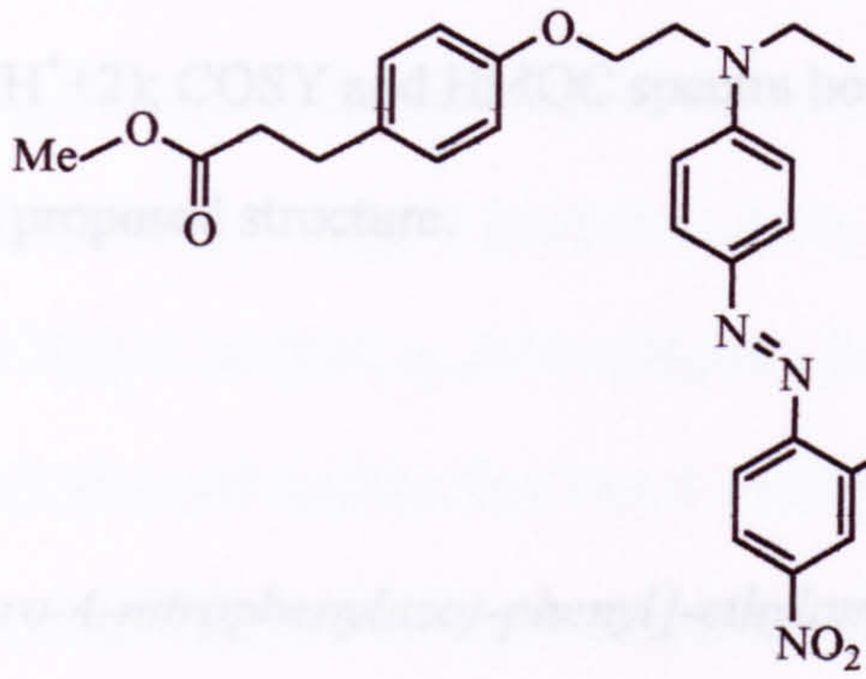
To a solution of **24** (200 mg, 0.41 mmol), triphenylphosphine (162 mg, 0.62 mmol, 1.5 eq) and diethyl azodicarboxylate (108 mg, 0.62 mmol, 1.5 eq) in toluene (20 ml) was added hexaethylene glycol (175 mg, 0.62 mmol, 1.5 eq). The reaction mixture was stirred for 5 days at room temperature after which TLC (methanol:ethyl acetate, 1:9) indicated that the acid had been entirely consumed. The toluene was removed at 50°C under reduced pressure and the product eluted by gradient flash column chromatography (ethyl acetate:hexane, 4:1; methanol:ethyl acetate, 1:9) to give **50** as a dark red oil, (209 mg, 0.28 mmol, 68%). ν_{max}(Nujol)/cm^{-1} 3463, 1731, 1598, 1513, 1337; δ_H(300 MHz; $CDCl_3$) 8.36 (1 H, d, *J* 2.5, ArC**H**), 8.12 (1 H, dd, *J* 9.0, 2.5, ArC**H**), 7.93 (2 H, d, J_{AB} 9.2, ArC**H**), 7.76 (1 H, d, *J* 9.0, ArC**H**), 7.20 (2 H, d, J_{AB} 8.7, ArC**H**), 6.84 (2 H, d, J_{AB} 8.7, ArC**H**), 6.81 (2 H, d, J_{AB} 9.2, ArC**H**), 4.24 (2 H, t, *J* 4.8, C$\mathbf{H}_2$), 4.17 (2 H, t, *J* 5.8, ArOC$\mathbf{H}_2$CH$_2$), 3.85 (2H, t, *J* 5.8, ArOCH$_2$C$\mathbf{H}_2$), 3.73-3.58 (26 H, m, C$\mathbf{H}_2$), 2.76 (1 H, s, O**H**), 1.29 (3 H, t, *J* 7.1, CH$_2$**Me**); δ_C(75 MHz; $CDCl_3$) 172.2 (C), 157.9 (C), 153.5 (C), 152.1 (C), 147.5 (C), 144.7 (C), 134.3 (C), 130.9 (2 CH), 127.4 (CH), 127.0 (C), 126.4 (CH), 123.0 (CH), 118.4 (2 CH), 114.9 (2 CH), 111.9 (2 CH), 72.9 (CH_2), 71.0 (CH_2), 70.9 (6CH_2), 70.7 (CH_2), 69.4 (CH_2), 65.7 (CH_2), 64.4 (CH_2), 62.1 (CH_2), 50.3 (CH_2), 46.7 (CH_2), 40.6 (CH_2), 12.7 (CH_3); *m/z* (EI) 746 (M^+), 748 (M^++2); (CI) 747 (MH^+), 749 (MH^++2) (Found: M^+, 746.2914. $C_{36}H_{47}{}^{35}ClN_4O_{11}$ requires M^+, 746.2930); HMQC spectra exhibited a good correlation with the proposed structure.

3-(4-Hydroxyphenyl)-propionic acid methyl ester, **43**.



To a solution of 3-(4-hydroxyphenyl)-propionic acid (1.0 g, 6.0 mmol) dissolved in methanol (100 ml), 4 drops of concentrated sulphuric acid were carefully added. The reaction mixture was refluxed for 3 hours after which TLC (ethyl acetate:hexane, 2:3) indicated complete conversion of the starting material. The reaction mixture was allowed to cool to room temperature and the methanol removed under reduced pressure. The product was firstly precipitated from diethyl ether and hexane at –78°C and then further purified by careful recrystallisation at –20°C from diethyl ether, cyclohexane and hexane to give **43** as white needles, (594 mg, 3.3 mmol, 55%). Alternatively the product could be isolated *via* reduced pressure distillation on a larger scale at 148°C (0.2 bar) to give a comparative yield, (66%). Isolation of the product *via* flash column chromatography (ethyl acetate:hexane, 1:4) gave an improved yield of 94%. mp 36-37°C (diethyl ether/cyclohexane); (Found: C, 67.00; H, 6.77. $C_{10}H_{12}O_3$ requires C, 66.65; H, 6.71%); ν_{max}(Nujol)/cm^{-1} 3398, 1715, 1614; δ_H(300 MHz; $CDCl_3$) 7.03 (2 H, d, J_{AB} 8.6, ArC**H**), 6.75 (2 H, d, J_{AB} 8.6, ArC**H**), 6.14 (1 H, s, ArO**H**), 3.67 (3 H, s, CO_2**Me**) 2.87 (2 H, t, *J* 7.8, $ArCH_2CH_2$), 2.60 (2 H, t, *J* 7.8, $ArCH_2CH_2$); δ_C(75 MHz; $CDCl_3$) 174.7 (C), 154.7 (C), 132.6 (C), 129.8 (2 CH), 115.8 (2 CH), 52.3 (CH_3), 36.5 (CH_2), 30.5 (CH_2); *m/z* (EI) 180 (M^+); (CI) 181 (MH^+), 198 (MNH_4^+); COSY spectra exhibited a good correlation with the proposed structure.

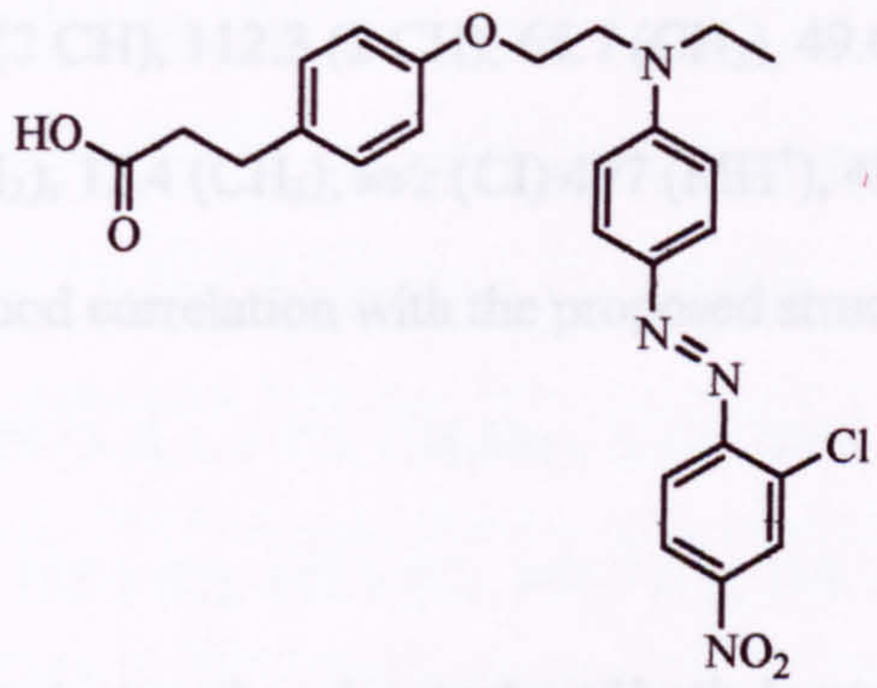
3-[4-(2-{[4-(2-Chloro-4-nitrophenylazo)-phenyl]-ethylamino}-ethoxy)-phenyl]-propionic acid methyl ester, **44**.[55-57]



To a solution of **43** (3 g, 16.7 mmol), triphenylphosphine (4.37 g, 16.7 mmol) and diethyl azodicarboxylate (2.59 ml, 16.7 mmol) in toluene (250 ml) at 80°C was added disperse red (5.2 g, 15.0 mmol, 0.9 eq). The reaction mixture was stirred at 80°C for 6 hours after which time TLC (ethyl acetate:hexane, 4:1) indicated that the disperse red had been entirely consumed. The toluene was then removed at 50°C under reduced pressure and the product isolated by flash column chromatography (ethyl acetate:hexane, 2:3) to give **44** as a purple powder, (4.43 g, 8.67 mmol, 52%). mp 116-118°C (ethyl acetate/hexane); (Found: C, 61.09; H, 5.32; N, 10.92. $C_{26}H_{27}ClN_4O_5$ requires C, 61.11; H, 5.33; N, 10.96%); ν_{max}(Nujol)/cm^{-1} 1734, 1599, 1512, 1336; δ_H(300 MHz; $CDCl_3$) 8.37 (1 H, d, *J* 2.4, ArC**H**), 8.13 (1 H, dd, *J* 8.9, 2.4, ArC**H**), 7.94 (2 H, d, J_{AB} 8.9, ArC**H**), 7.77 (1 H, d, *J* 8.9, ArC**H**), 7.11 (2 H, d, J_{AB} 8.9, ArC**H**), 6.81 (4 H, d, J_{AB} 8.9, ArC**H**), 4.17 (2 H, t, *J* 5.7, ArOC**H**$_2$CH$_2$), 3.85 (2 H, t, *J* 5.7, ArOCH$_2$C**H**$_2$), 3.72-3.59 (5 H, m, CO$_2$**Me,** C**H**$_2$Me), 2.89 (2 H, t, *J* 7.6, CH$_2$C**H**$_2$CO$_2$), 2.59 (2 H, t, *J* 7.6, C**H**$_2$CH$_2$CO$_2$), 1.29 (3 H, t, *J* 7.1, CH$_2$**Me**); δ_C(75 MHz; $CDCl_3$) 173.3 (C), 156.8 (C), 153.0 (C), 151.6 (C), 147.0 (C), 144.3

(C), 133.9 (C), 133.2 (C), 129.3 (CH), 126.9 (CH), 126.0 (CH), 122.6 (CH), 118.0 (CH), 114.4 (CH), 111.5 (CH), 65.2 (CH_2), 51.6 (CH_3), 50.0 (CH_2), 46.3 (CH_2), 35.9 (CH_2), 30.0 (CH_2), 12.3 (CH_3); *m/z* (EI) 510 (M^+), 512 (M^++2); (CI) 511 (MH^+), 513 (MH^++2); COSY and HMQC spectra both exhibited a good correlation with the proposed structure.

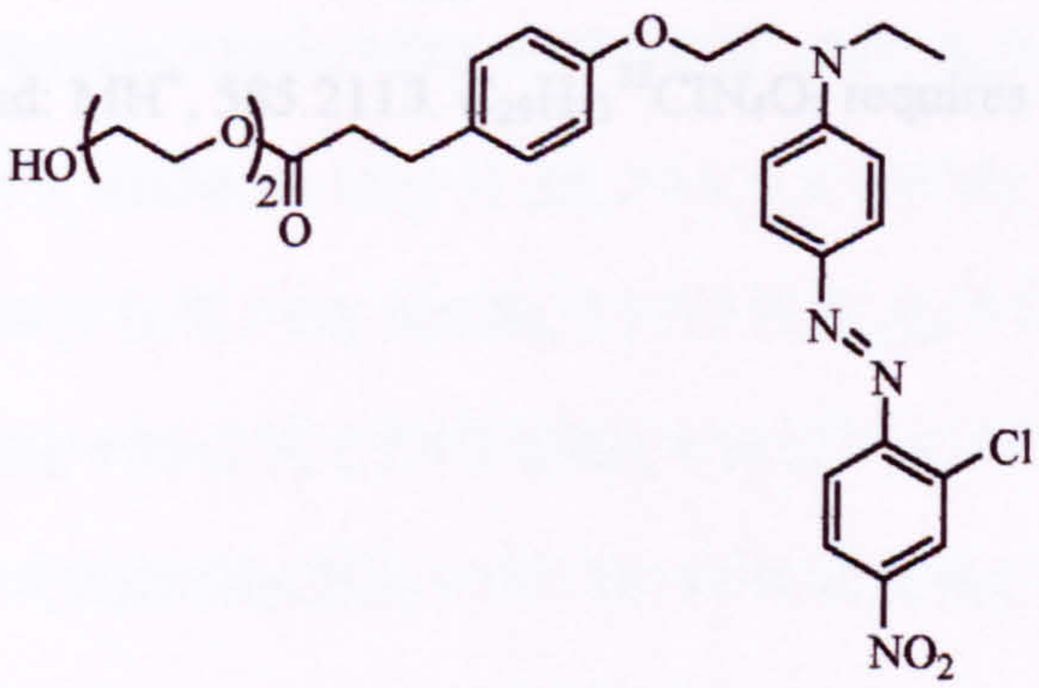
3-[4-(2-{[4-(2-Chloro-4-nitrophenylazo)-phenyl]-ethylamino}-ethoxy)-phenyl]-propionic acid, **45**.[58]



To a solution of **44** (2 g, 3.92 mmol) in tetrahydrofuran (150 ml) and water (5 ml) was added lithium hydroxide monohydrate (247 mg, 5.88 mmol, 1.5 eq). The reaction mixture was stirred at room temperature for 12 hours and then 40°C for 3 hours after which TLC (ethyl acetate:hexane, 4:1) indicated that the starting material had been consumed. This was then allowed to cool to room temperature, concentrated at 30°C under reduced pressure, diluted with dichloromethane (40 ml), washed with HCl (2 M, 3 x 30 ml) and water (3 x 30 ml), dried ($MgSO_4$) and concentrated in *vacuo*. The product was eluted by flash column chromatography (ethyl acetate:hexane, 3:2) to give **45** as a dark red oil, (1.46 g, 2.94 mmol, 75%). mp 177-179°C (ethyl acetate/hexane); (Found: C,

60.33; H, 5.08; N, 11.13. $C_{25}H_{25}ClN_4O_5$ requires C, 60.42; H, 5.07; N, 11.27%); ν_{max}(Nujol)/cm^{-1} 1698, 1603, 1511, 1341; δ_H(300 MHz; $CDCl_3$) 8.37 (1 H, d, *J* 2.5, ArC**H**), 8.18 (1 H, dd, *J* 9.0, 2.5, ArC**H**), 7.80 (2 H, d, J_{AB} 9.2, ArC**H**), 7.73 (1 H, d, *J* 9.0, ArC**H**), 7.06 (2 H, d, J_{AB} 8.6, ArC**H**), 6.91 (2 H, d, J_{AB} 9.2, ArC**H**), 6.78 (2 H, d, J_{AB} 8.6, ArC**H**), 4.10 (2 H, t, *J* 5.4, ArOC**H**$_2$CH$_2$), 3.81 (2 H, t, *J* 5.4, ArOCH$_2$C**H**$_2$), 3.56 (2 H, q, *J* 7.0, C**H**$_2$Me), 2.68 (2 H, t, *J* 7.5, CH$_2$C**H**$_2$CO$_2$), 2.41 (2 H, t, *J* 7.5, C**H**$_2$CH$_2$CO$_2$), 1.14 (3 H, t, *J* 7.0, CH$_2$**Me**); δ_C(75 MHz; $CDCl_3$) 174.3 (C), 156.8 (C), 152.7 (C), 152.5 (C), 147.1 (C), 143.7 (C), 133.9 (C), 132.7 (C), 129.6 (2 CH), 127.1 (CH), 126.1 (CH), 123.7 (CH), 118.4 (2 CH), 114.5 (2 CH), 112.3 (2 CH), 65.7 (CH$_2$), 49.6 (CH$_2$), 45.8 (CH$_2$), 36.6 (CH$_2$), 30.2 (CH$_2$), 12.4 (CH$_3$); *m/z* (CI) 497 (MH$^+$), 499 (MH$^+$+2); COSY spectra exhibited a good correlation with the proposed structure.

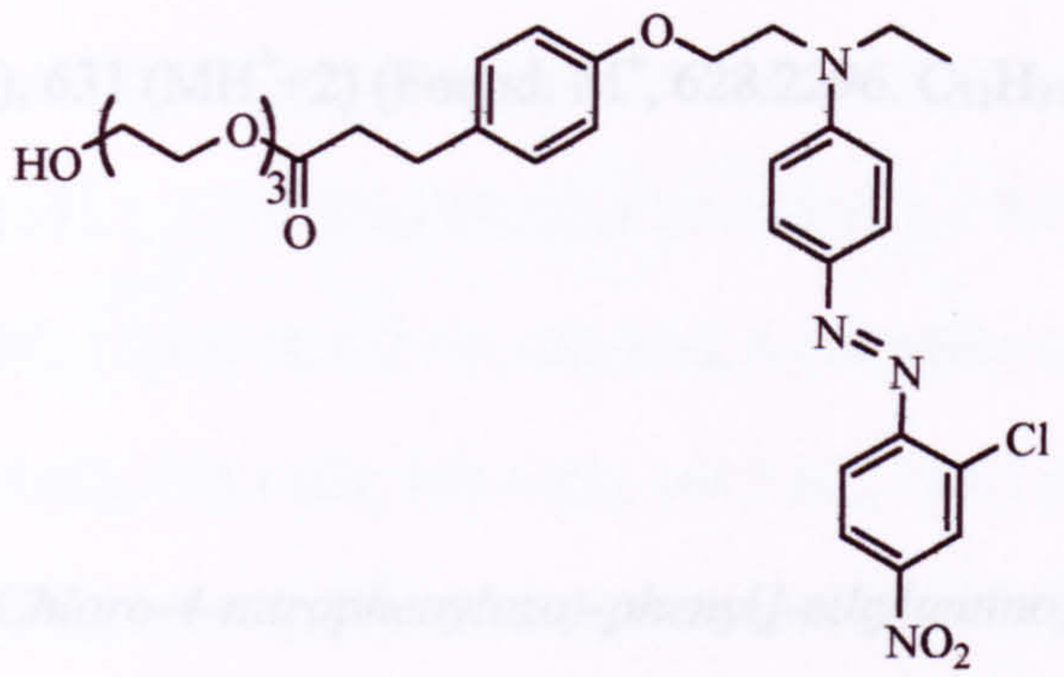
3-[4-(2-{[4-(2-Chloro-4-nitrophenylazo)-phenyl]-ethylamino}-ethoxy)-phenyl]-propionic acid 2-(2-hydroxyethoxy)-ethyl ester, **51**.[55-57]



To a solution of **45** (200 mg, 0.40 mmol), triphenylphosphine (158 mg, 0.60 mmol, 1.5 eq) and diethyl azodicarboxylate (105 mg, 0.60 mmol, 1.5 eq) in

dichloromethane (20 ml) diethylene glycol (64 mg, 0.60 mmol, 1.5 eq) was added. The reaction mixture was stirred for 3 hours at room temperature after which TLC (methanol:ethyl acetate, 1:9) indicated that the acid had been entirely consumed. The toluene was removed at 50°C under reduced pressure and the product eluted by gradient flash column chromatography (ethyl acetate:hexane, 4:1; methanol:ethyl acetate, 1:9) to give **51** as a dark red oil, (174 mg, 0.30 mmol, 74%). ν_{max}(Nujol)/cm^{-1} 3443, 1731, 1599, 1513, 1336; δ_H(300 MHz; $CDCl_3$) 8.36 (1 H, d, *J* 2.5, ArC**H**), 8.13 (1 H, dd, *J* 9.0, 2.5, ArC**H**), 7.93 (2 H, d, J_{AB} 9.1, ArC**H**), 7.76 (1 H, d, *J* 9.0, ArC**H**), 7.11 (2 H, d, J_{AB} 8.5, ArC**H**), 6.81 (2 H, d, J_{AB} 8.5, ArC**H**), 6.80 (2 H, d, J_{AB} 9.1, ArC**H**), 4.23 (2 H, t, *J* 4.6, C$\mathbf{H}_2$), 4.16 (2 H, t, *J* 5.6, ArOC$\mathbf{H}_2$CH$_2$), 3.84 (2 H, t, *J* 5.6, ArOCH$_2$C$\mathbf{H}_2$), 3.73-3.55 (8 H, m, C$\mathbf{H}_2$), 2.89 (2 H, t, *J* 7.6, CH$_2$C$\mathbf{H}_2$CO$_2$), 2.63 (2 H, t, *J* 7.6, C$\mathbf{H}_2$CH$_2$CO$_2$), 2.12 (1 H, s, O**H**), 1.29 (3 H, t, *J* 7.0, CH$_2$**Me**); δ_C(75 MHz; $CDCl_3$) 173.3 (C), 157.3 (C), 153.5 (C), 152.1 (C), 147.5 (C), 144.7 (C), 134.3 (C), 133.5 (C), 129.8 (2 CH), 127.4 (CH), 126.4 (CH), 123.0 (CH), 118.4 (2 CH), 114.8 (2 CH), 111.9 (2 CH), 72.7 (CH_2), 69.5 (CH_2), 65.7 (CH_2), 63.9 (CH_2), 62.1 (CH_2), 50.4 (CH_2), 46.7 (CH_2), 36.4 (CH_2), 30.4 (CH_2), 12.7 (CH_3); *m/z* (CI) 585 (MH^+), 587 (MH^++2) (Found: MH^+, 585.2113. $C_{29}H_{33}{}^{35}ClN_4O_7$ requires MH^+, 585.2116);

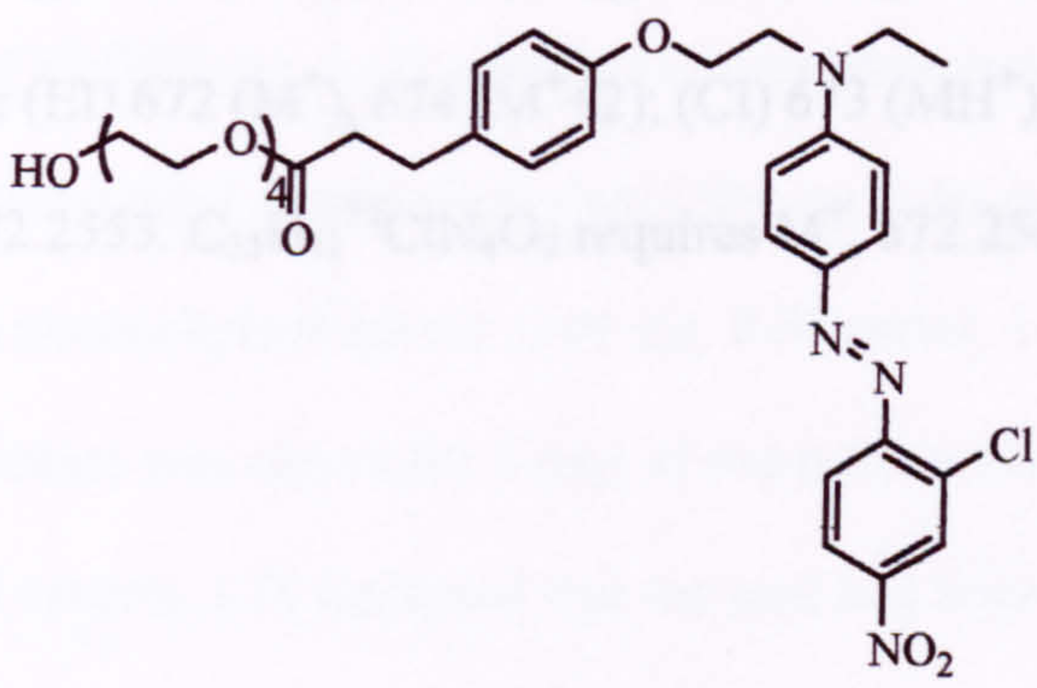
3-[4-(2-{[4-(2-Chloro-4-nitrophenylazo)-phenyl]-ethylamino}-ethoxy)-phenyl]-propionic acid 2-[2-(2-hydroxyethoxy)-ethoxy]-ethyl ester, **52**.[55-57]



To a solution of **45** (200 mg, 0.40 mmol), triphenylphosphine (158 mg, 0.60 mmol, 1.5 eq) and diethyl azodicarboxylate (104 mg, 0.60 mmol, 1.5 eq) in toluene (20 ml) triethylene glycol (91 mg, 0.60 mmol, 1.5 eq) was added. The reaction mixture was stirred for 5 days at room temperature after which TLC (methanol:ethyl acetate, 1:9) indicated that the acid had been entirely consumed. The toluene was removed at 50°C under reduced pressure and the product eluted by gradient flash column chromatography (ethyl acetate:hexane, 4:1; methanol:ethyl acetate, 1:9) to give **52** as a dark red oil, (175 mg, 0.28 mmol, 69%). ν_{max}(Nujol)/cm^{-1} 3456, 1736, 1599, 1514, 1337; δ_H(300 MHz; $CDCl_3$) 8.36 (1 H, d, *J* 2.5, ArC**H**), 8.13 (1 H, dd, *J* 9.0, 2.5, ArC**H**), 7.93 (2 H, d, J_{AB} 8.9, ArC**H**), 7.76 (1 H, d, *J* 9.0, ArC**H**), 7.12 (2 H, d, J_{AB} 8.9, ArC**H**), 6.81 (4 H, d, J_{AB} 8.9, ArC**H**), 4.23 (2 H, t, *J* 4.7, C**H**$_2$), 4.16 (2 H, t, *J* 5.7, ArOC**H**$_2$CH$_2$), 3.85 (2 H, t, *J* 5.7, ArOCH$_2$C**H**$_2$), 3.73-3.58 (12 H, m, C**H**$_2$), 2.89 (2 H, t, *J* 7.6, CH$_2$C**H**$_2$CO$_2$), 2.63 (2 H, t, *J* 7.6, C**H**$_2$CH$_2$CO$_2$), 2.41 (1 H, s, O**H**), 1.29 (3 H, t, *J* 7.0, CH$_2$**Me**); δ_C(75 MHz; $CDCl_3$) 173.3 (C), 157.3 (C), 153.5 (C), 152.1 (C), 147.5 (C), 144.7 (C), 134.3 (C), 133.6 (C), 129.8 (2 CH), 127.4 (CH), 126.4

(CH), 123.0 (CH), 118.4 (2 CH), 114.8 (2 CH), 112.0 (2 CH), 72.9 (CH_2), 70.9 (CH_2), 70.7 (CH_2), 69.5 (CH_2), 65.7 (CH_2), 63.8 (CH_2), 62.1 (CH_2), 50.4 (CH_2), 46.7 (CH_2), 36.4 (CH_2), 30.4 (CH_2), 12.7 (CH_3); *m/z* (EI) 628 (M^+), 630 (M^++2); (CI) 629 (MH^+), 631 (MH^++2) (Found: M^+, 628.2296. $C_{31}H_{37}{}^{35}ClN_4O_8$ requires M^+, 628.2300);

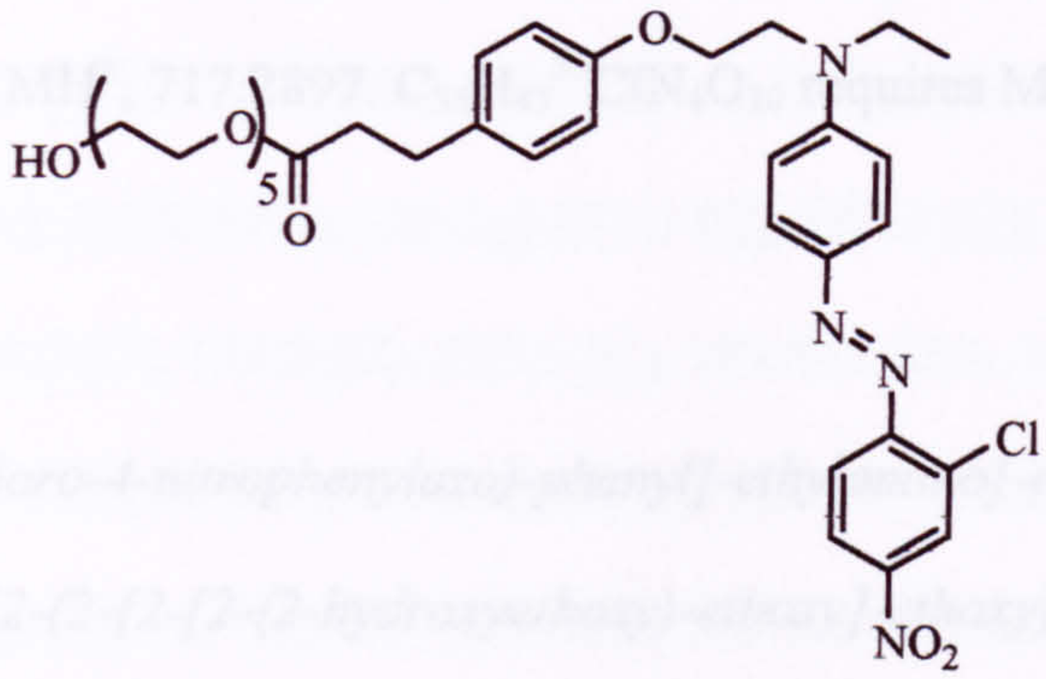
3-[4-(2-{[4-(2-Chloro-4-nitrophenylazo)-phenyl]-ethylamino}-ethoxy)-phenyl]-propionic acid 2-{2-[2-(2-hydroxyethoxy)-ethoxy]-ethoxy}-ethyl ester, **53**.[55-57]



To a solution of **45** (200 mg, 0.40 mmol), triphenylphosphine (158 mg, 0.60 mmol, 1.5 eq) and diethyl azodicarboxylate (104 mg, 0.60 mmol, 1.5 eq) in toluene (20 ml) tetraethylene glycol (117 mg, 0.60 mmol, 1.5 eq) was added. The reaction mixture was stirred for 5 days at room temperature after which TLC (methanol:ethyl acetate, 1:9) indicated that the acid had been entirely consumed. The toluene was removed at 50°C under reduced pressure and the product eluted by gradient flash column chromatography (ethyl acetate:hexane, 4:1; methanol:ethyl acetate, 1:9) to give **53** as a dark red oil, (190 mg, 0.28 mmol, 70%). ν_{max}(Nujol)/cm^{-1} 3457, 1731, 1599, 1519, 1337; δ_H(300 MHz; $CDCl_3$)

8.35 (1 H, d, *J* 2.5, ArC**H**), 8.11 (1 H, dd, *J* 9.0, 2.5, ArC**H**), 7.92 (2 H, d, J_{AB} 9.2, ArC**H**), 7.76 (1 H, d, *J* 9.0, ArC**H**), 7.12 (2 H, d, J_{AB} 8.5, ArC**H**), 6.81 (2 H, d, J_{AB} 8.5, ArC**H**), 6.80 (2 H, d, J_{AB} 9.2, ArC**H**), 4.23 (2 H, t, *J* 4.7, C$\mathbf{H}_2$), 4.16 (2 H, t, *J* 5.7, ArOC$\mathbf{H}_2$CH$_2$), 3.84 (2 H, t, *J* 5.7, ArOCH$_2$C$\mathbf{H}_2$), 3.73-3.58 (16 H, m, C$\mathbf{H}_2$), 2.89 (2 H, t, *J* 7.7, CH$_2$C$\mathbf{H}_2$CO$_2$), 2.62 (2 H, t, *J* 7.7, C$\mathbf{H}_2$CH$_2$CO$_2$), 2.61 (1 H, s, O**H**), 1.29 (3 H, t, *J* 7.0, CH$_2$**Me**); δ_C(75 MHz; $CDCl_3$) 173.3 (C), 157.3 (C), 153.4 (C), 152.1 (C), 147.4 (C), 144.7 (C), 134.3 (C), 133.6 (C), 129.8 (2 CH), 127.4 (CH), 126.4 (CH), 123.0 (CH), 118.4 (2 CH), 114.8 (2 CH), 111.9 (2 CH), 72.9 (CH_2), 71.0 (CH_2), 70.9 (2CH_2), 70.7 (CH_2), 69.5 (CH_2), 65.7 (CH_2), 63.9 (CH_2), 62.1 (CH_2), 50.4 (CH_2), 46.7 (CH_2), 36.4 (CH_2), 30.4 (CH_2), 12.7 (CH_3); *m/z* (EI) 672 (M^+), 674 (M^++2); (CI) 673 (MH^+), 675 (MH^++2) (Found: M^+, 672.2553. $C_{33}H_{41}{}^{35}ClN_4O_9$ requires M^+, 672.2562);

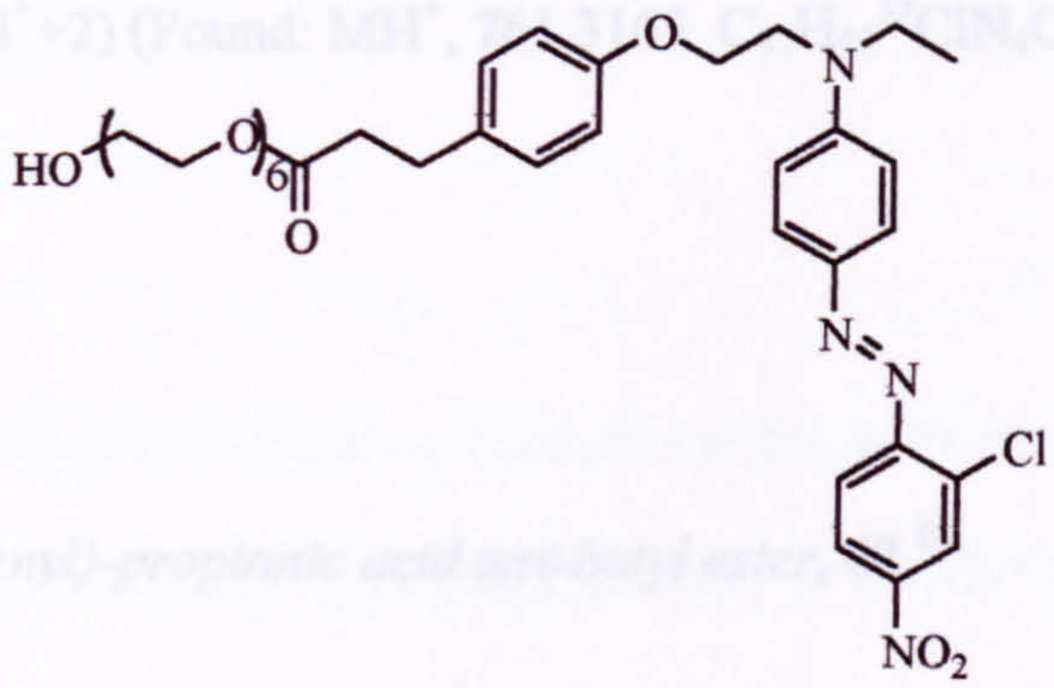
3-[4-(2-{[4-(2-Chloro-4-nitrophenylazo)-phenyl]-ethyl-amino}-ethoxy)-phenyl]-propionic acid 2-(2-{2-[2-(2-hydroxyethoxy)-ethoxy]-ethoxy}-ethoxy)-ethyl ester, **54**.[55-57]



To a solution of **45** (200 mg, 0.40 mmol), triphenylphosphine (158 mg, 0.60 mmol, 1.5 eq) and diethyl azodicarboxylate (104 mg, 0.60 mmol, 1.5 eq) in toluene (20 ml) pentaethylene glycol (144 mg, 0.60 mmol, 1.5 eq) was added. The reaction mixture was stirred for 5 days at room temperature after which TLC (methanol:ethyl acetate, 1:9) indicated that the acid had been entirely consumed. The toluene was removed at 50°C under reduced pressure and the product eluted by gradient flash column chromatography (ethyl acetate:hexane, 4:1; methanol:ethyl acetate1:9) to give **54** as a dark red oil, (190 mg, 0.26 mmol, 66%). ν_{max}(Nujol)/cm^{-1} 3477, 1731, 1599, 1519, 1337; δ_H(300 MHz; $CDCl_3$) 8.37 (1 H, d, *J* 2.5, ArC**H**), 8.13 (1 H, dd, *J* 9.1, 2.5, ArC**H**), 7.93 (2 H, d, J_{AB} 8.8, ArC**H**), 7.77 (1 H, d, *J* 9.1, ArC**H**), 7.12 (2 H, d, J_{AB} 8.8, ArC**H**), 6.81 (4 H, d, J_{AB} 8.8, ArC**H**), 4.22 (2 H, t, *J* 4.8, C$\mathbf{H}_2$), 4.16 (2 H, t, *J* 5.8, ArOC$\mathbf{H}_2$CH$_2$), 3.85 (2 H, t, *J* 5.8, ArOCH$_2$C$\mathbf{H}_2$), 3.73-3.58 (20 H, m, C$\mathbf{H}_2$), 2.89 (2 H, t, *J* 7.7, CH$_2$C$\mathbf{H}_2$CO$_2$), 2.68 (1 H, s, O**H**), 2.62 (2 H, t, *J* 7.7, C$\mathbf{H}_2$CH$_2$CO$_2$), 1.29 (3 H, t, *J* 7.1, CH$_2$**Me**); δ_C(75 MHz; $CDCl_3$) 173.3 (C), 157.3 (C), 153.5 (C), 152.1 (C),

147.5 (C), 144.7 (C), 134.3 (C), 133.6 (C), 129.8 (2 CH), 127.4 (CH), 126.4 (CH), 123.0 (CH), 118.4 (2 CH), 114.8 (2 CH), 111.9 (2 CH), 72.9 (CH_2), 70.9 (5CH_2), 70.7 (CH_2), 69.5 (CH_2), 65.7 (CH_2), 64.0 (CH_2), 62.1 (CH_2), 50.4 (CH_2), 46.7 (CH_2), 36.4 (CH_2), 30.4 (CH_2), 12.7 (CH_3); *m/z* (CI) 717 (MH^+), 719 (MH^++2) (Found: MH^+, 717.2897. $C_{35}H_{43}{}^{35}ClN_4O_{10}$ requires MH^+, 717.2902);

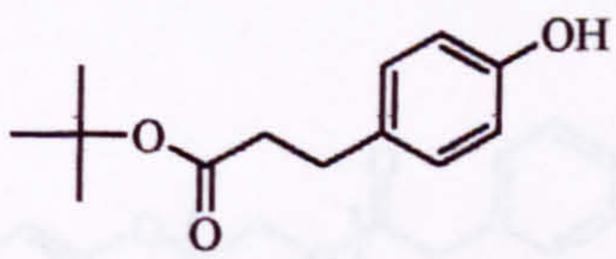
3-[4-(2-{[4-(2-Chloro-4-nitrophenylazo)-phenyl]-ethylamino}-ethoxy)-phenyl]-propionic acid 2-[2-(2-{2-[2-(2-hydroxyethoxy)-ethoxy]-ethoxy}-ethoxy)-ethoxy]-ethyl ester, **55**.[55-57]



To a solution of **45** (200 mg, 0.40 mmol), triphenylphosphine (158 mg, 0.60 mmol, 1.5 eq) and diethyl azodicarboxylate (104 mg, 0.60 mmol, 1.5 eq) in toluene (20 ml) hexaethylene glycol (170 mg, 0.60 mmol, 1.5 eq) was added. The reaction mixture was stirred for 5 days at room temperature after which TLC (methanol:ethyl acetate, 1:9) indicated that the acid had been entirely consumed. The toluene was removed at 50°C under reduced pressure and the product eluted by gradient flash column chromatography (ethyl acetate:hexane, 4:1; methanol:ethyl acetate, 1:9) to give **55** as a dark red oil, (184 mg, 0.24 mmol, 60%). ν_{max}(Nujol)/cm^{-1} 3455, 1732, 1600, 1519, 1337; δ_H(300 MHz; $CDCl_3$)

8.37 (1 H, d, *J* 2.3, ArC**H**), 8.13 (1 H, dd, *J* 9.0, 2.3, ArC**H**), 7.94 (2 H, d, J_{AB} 8.9, ArC**H**), 7.77 (1 H, d, *J* 9.0, ArC**H**), 7.12 (2 H, d, J_{AB} 8.9, ArC**H**), 6.81 (4 H, d, J_{AB} 8.9, ArC**H**), 4.22 (2 H, t, *J* 4.7, C$\mathbf{H}_2$), 4.17 (2 H, t, *J* 5.7, ArOC$\mathbf{H}_2$CH$_2$), 3.85 (2 H, t, *J* 5.7, ArOCH$_2$C$\mathbf{H}_2$), 3.73-3.58 (24 H, m, C$\mathbf{H}_2$Me), 2.89 (2 H, t, *J* 7.7, CH$_2$C$\mathbf{H}_2$CO$_2$), 2.79 (1 H, s, O**H**), 2.62 (2 H, t, *J* 7.7, C$\mathbf{H}_2$CH$_2$CO$_2$), 1.29 (3 H, t, *J* 7.1, CH$_2$**Me**); δ_C(75 MHz; $CDCl_3$) 173.3 (C), 157.3 (C), 153.5 (C), 152.1 (C), 147.5 (C), 144.7 (C), 134.3 (C), 133.6 (C), 129.8 (2 CH), 127.4 (CH), 126.4 (CH), 123.0 (CH), 118.4 (2 CH), 114.8 (2 CH), 111.9 (2 CH), 73.0 (CH_2), 71.0 (CH_2), 70.9 (6CH_2), 70.7 (CH_2), 69.5 (CH_2), 65.7 (CH_2), 64.0 (CH_2), 62.1 (CH_2), 50.4 (CH_2), 46.7 (CH_2), 36.4 (CH_2), 30.4 (CH_2), 12.7 (CH_3); *m/z* (CI) 761 (MH^+), 763 (MH^++2) (Found: MH^+, 761.3165. $C_{37}H_{49}{}^{35}ClN_4O_{11}$ requires MH^+, 761.3165);

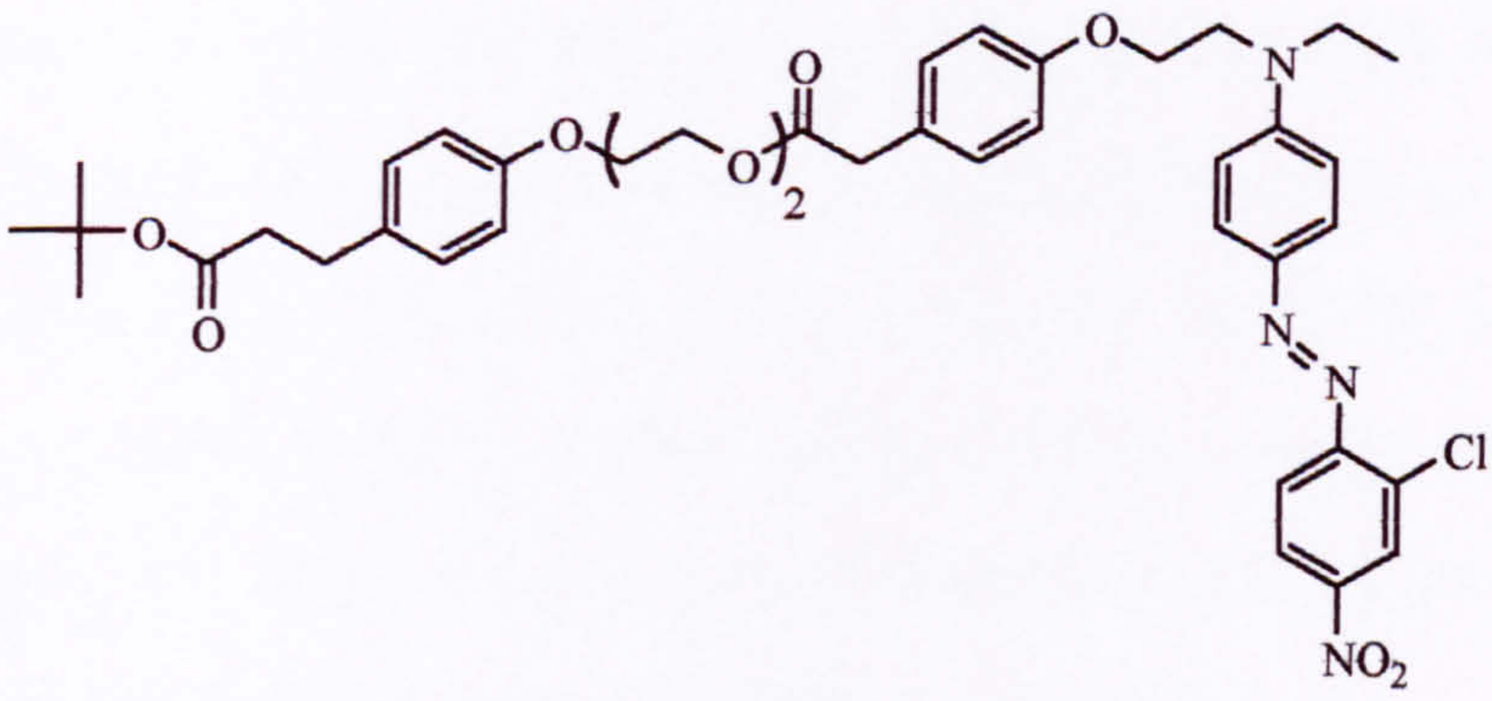
3-(4-Hydroxyphenyl)-propionic acid tert-butyl ester, **62**.[84]



To a solution of 3-(4-hydroxyphenyl) propionic acid (3.32 g, 20 mmol) in dimethylformamide (20 ml) was carefully added carbonyl diimidazole (3.24 g, 20 mmol). The reaction mixture was stirred at 40°C for 2 hours. DBU (6.08 ml, 40 mmol, 2 eq) and *tert*-butanol (3.70 ml, 50 mmol, 2.5 eq) were then added and the reaction mixture stirred at 65°C for 2 days after which time TLC (ethyl acetate:hexane, 1:4) indicated that the starting material had been consumed. The reaction mixture was cooled to room temperature, water (40 ml) added and the

product extracted with diethyl ether (3 x 20 ml). The organic fraction was dried ($MgSO_4$), concentrated under reduced pressure and the product isolated by flash column chromatography (ethyl acetate:hexane, 1:4) to give **62** as a clear colourless oil (2.89 g, 13 mmol, 65%). ν_{max}(Nujol)/cm^{-1} 3400, 1700, 1601, 1516; δ_H(300 MHz; $CDCl_3$) 7.05 (1 H, s, ArO**H**), 7.01 (2 H, d, J_{AB} 8.5, ArC**H**), 6.74 (2 H, d, J_{AB} 8.5, ArC**H**), 2.82 (2 H, t, *J* 7.6, $ArCH_2C\mathbf{H}_2$), 2.51 (2 H, t, *J* 7.6, $ArC\mathbf{H}_2CH_2$), 1.41 (9 H, s, C**Me**$_3$); δ_C(75 MHz; $CDCl_3$) 174.0 (C), 154.9 (C), 132.4 (C), 129.8 (2 CH), 115.8 (2 CH), 81.5 (C), 38.0 (CH_2), 30.7 (CH_2), 15.5 (CH_3); *m/z* (CI) 240 (MNH_4^+) (Found: MNH_4^+, 240.1597. $C_{13}H_{18}O_3$ requires MNH_4^+, 240.1600); COSY spectra exhibited a good correlation with the proposed structure.

3-{4-[2-(2-{2-[4-(2-{[4-(2-Chloro-4-nitrophenylazo)-phenyl]-ethylamino}-ethoxy)-phenyl]-acetoxy}-ethoxy)-ethoxy]-phenyl}-propionic acid tert-butyl ester, **63**.[55-57]

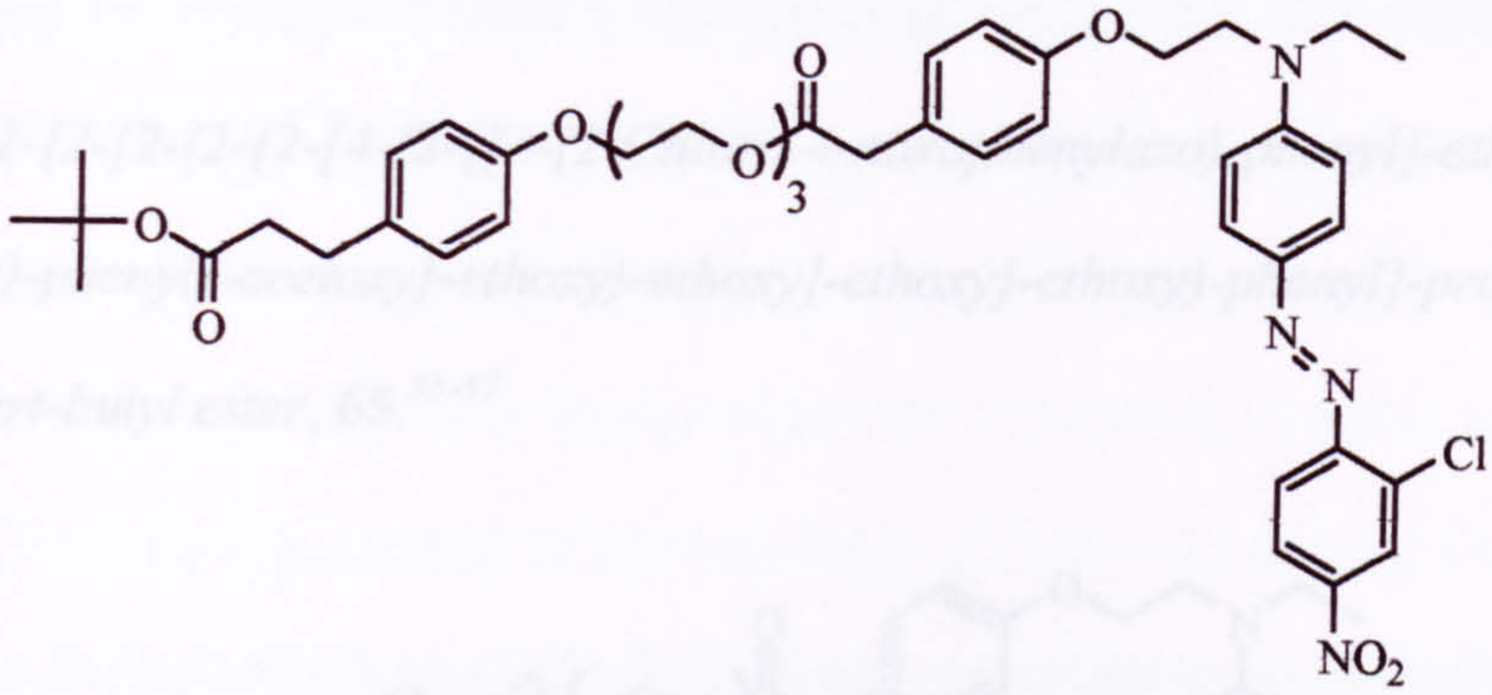


To a solution of **46** (144 mg, 0.25 mmol), triphenylphosphine (98 mg, 0.38 mmol, 1.5 eq) and diethyl azodicarboxylate (66 mg, 0.38 mmol, 1.5 eq) in dichloromethane (5 ml) was added *tert*-butyl ester **62** (111 mg, 0.50 mmol, 2.0

eq). The reaction mixture was stirred at room temperature for 5 hours after which time TLC (methanol:ethyl acetate, 1:9) indicated that the starting material had been consumed. The dichloromethane was removed under reduced pressure and the product isolated by gradient flash column chromatography (ethyl acetate:hexane, 4:1; methanol:ethyl acetate, 1:9) to give **63** as a dark red oil, (102 mg, 0.13 mmol, 53%). ν_{max}(Nujol)/cm^{-1} 1729, 1600, 1513, 1340; δ_H(300 MHz; $CDCl_3$) 8.39 (1 H, d, *J* 2.5, ArC**H**), 8.15 (1 H, dd, *J* 9.0, 2.5, ArC**H**), 7.95 (2 H, d, J_{AB} 9.2, ArC**H**), 7.78 (1 H, d, *J* 9.0, ArC**H**), 7.19 (2 H, d, J_{AB} 8.7, ArC**H**), 7.10 (2 H, d, J_{AB} 8.7, ArC**H**), 6.84-6.79 (6 H, m, ArC**H**), 4.27 (2 H, t, *J* 4.8, ArOC**H**$_2$CH$_2$), 4.16 (2 H, t, *J* 5.8, ArOC**H**$_2$CH$_2$), 4.07 (2 H, t, *J* 4.8, OCH$_2$C**H**$_2$O$_2$C), 3.87-3.74 (6 H, m, C**H**$_2$), 3.62 (2 H, q, *J* 7.2, C**H**$_2$Me), 3.58 (2 H, s, ArC**H**$_2$), 2.84 (2 H, t, *J* 7.6, CH$_2$C**H**$_2$CO$_2$), 2.50 (2 H, t, *J* 7.6, C**H**$_2$CH$_2$CO$_2$), 1.41 (9 H, s, C**Me**$_3$), 1.29 (3 H, t, *J* 7.2, CH$_2$**Me**); *m/z* (FAB) 774 (MH^+), 797 (MNa^+) (Found: MH^+, 775.3120. $C_{41}H_{47}{}^{35}ClN_4O_9$ requires MH^+, 775.3110); COSY spectra exhibited a good correlation with the proposed structure.

eq). The reaction mixture was stirred at room temperature for 5 hours after which time TLC (methanol:ethyl acetate, 1:9) indicated that the starting material had been consumed. The dichloromethane was removed under reduced pressure and the product isolated by gradient flash column chromatography (ethyl acetate:hexane, 4:1; methanol:ethyl acetate, 1:9) to give **63** as a dark red oil, (102 mg, 0.13 mmol, 53%). ν_{max}(Nujol)/cm^{-1} 1729, 1600, 1513, 1340; δ_H(300 MHz; $CDCl_3$) 8.39 (1 H, d, *J* 2.5, ArC**H**), 8.15 (1 H, dd, *J* 9.0, 2.5, ArC**H**), 7.95 (2 H, d, J_{AB} 9.2, ArC**H**), 7.78 (1 H, d, *J* 9.0, ArC**H**), 7.19 (2 H, d, J_{AB} 8.7, ArC**H**), 7.10 (2 H, d, J_{AB} 8.7, ArC**H**), 6.84-6.79 (6 H, m, ArC**H**), 4.27 (2 H, t, *J* 4.8, ArOC**H**$_2$CH$_2$), 4.16 (2 H, t, *J* 5.8, ArOC**H**$_2$CH$_2$), 4.07 (2 H, t, *J* 4.8, OCH$_2$C**H**$_2$O$_2$C), 3.87-3.74 (6 H, m, C**H**$_2$), 3.62 (2 H, q, *J* 7.2, C**H**$_2$Me), 3.58 (2 H, s, ArC**H**$_2$), 2.84 (2 H, t, *J* 7.6, CH$_2$C**H**$_2$CO$_2$), 2.50 (2 H, t, *J* 7.6, C**H**$_2$CH$_2$CO$_2$), 1.41 (9 H, s, C**Me**$_3$), 1.29 (3 H, t, *J* 7.2, CH$_2$**Me**); *m/z* (FAB) 774 (MH$^+$), 797 (MNa$^+$) (Found: MH$^+$, 775.3120. $C_{41}H_{47}{}^{35}ClN_4O_9$ requires MH$^+$, 775.3110); COSY spectra exhibited a good correlation with the proposed structure.

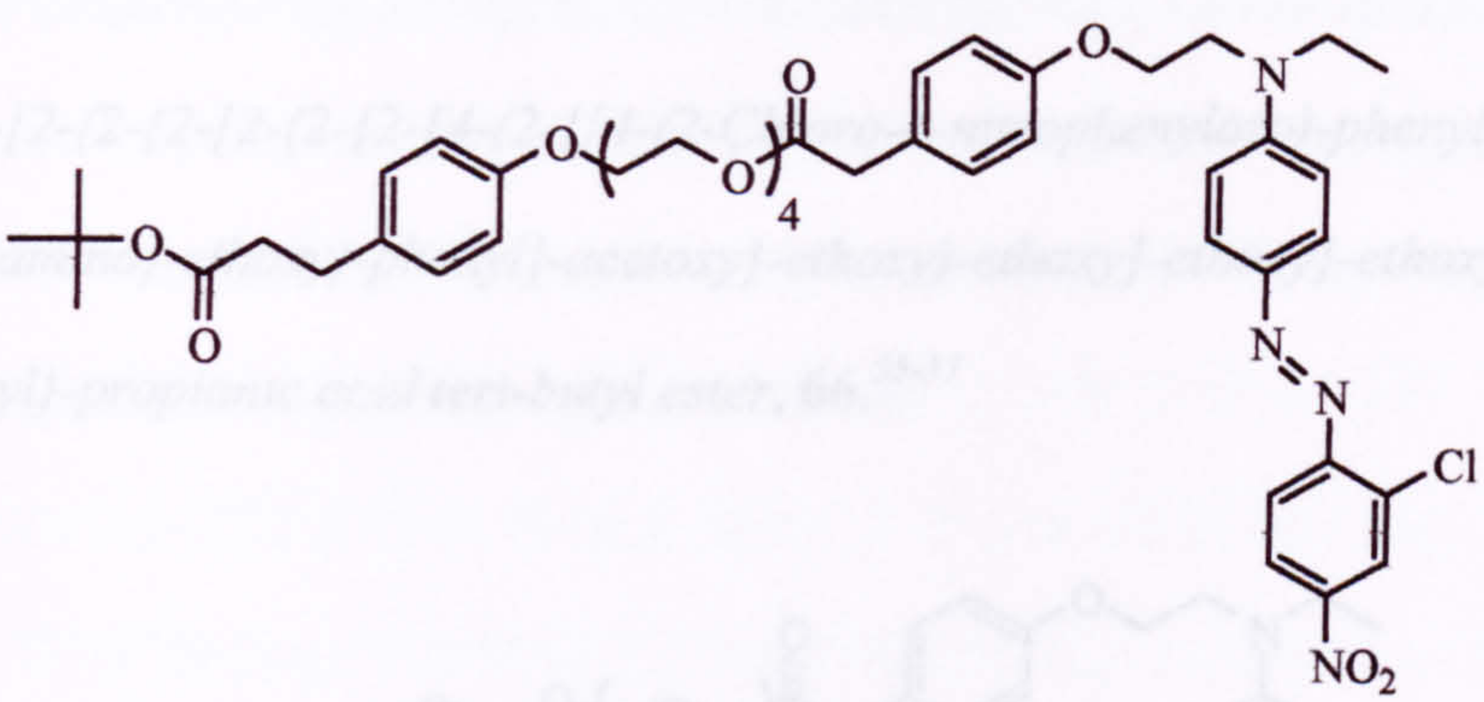
3-(4-{2-[2-(2-{2-[4-(2-{[4-(2-Chloro-4-nitrophenylazo)-phenyl]-ethylamino}-ethoxy)-phenyl]-acetoxy}-ethoxy)-ethoxy]-ethoxy}-phenyl)-propionic acid tert-butyl ester, **64**.[55-57]



To a solution of **47** (168 mg, 0.27 mmol), triphenylphosphine (108 mg, 0.41 mmol, 1.5 eq) and diethyl azodicarboxylate (71 mg, 0.41 mmol, 1.5 eq) in dichloromethane (5 ml) was added *tert*-butyl ester **62** (121 mg, 0.55 mmol, 2.0 eq). The reaction mixture was stirred at room temperature for 5 hours after which time TLC (methanol:ethyl acetate, 1:9) indicated that the starting material had been consumed. The dichloromethane was removed under reduced pressure and the product isolated by gradient flash column chromatography (ethyl acetate:hexane, 4:1; methanol:ethyl acetate, 1:9) to give **64** as a dark red oil, (123 mg, 0.15 mmol, 55%). ν_{max}(Nujol)/cm^{-1} 1735, 1601, 1514, 1340; δ_H(300 MHz; $CDCl_3$) 8.39 (1 H, d, *J* 2.5, ArC**H**), 8.15 (1 H, dd, *J* 9.0, 2.5, ArC**H**), 7.95 (2 H, d, J_{AB} 9.2, ArC**H**), 7.78 (1 H, d, *J* 9.0, ArC**H**), 7.19 (2 H, d, J_{AB} 8.7, ArC**H**), 7.09 (2 H, d, J_{AB} 8.7, ArC**H**), 6.85-6.79 (6 H, m, ArC**H**), 4.24 (2 H, t, *J* 5.3, ArOC**H**$_2$CH$_2$), 4.17 (2 H, t, *J* 5.7, ArOC**H**$_2$CH$_2$), 4.09 (2 H, t, *J* 5.1, OCH$_2$C**H**$_2$O$_2$C), 3.87-3.81 (4 H, m, CH$_2$), 3.71-3.61 (8 H, m, C**H**$_2$), 3.58 (2 H, s, ArC**H**$_2$), 2.83 (2 H, t, *J* 7.7, CH$_2$C**H**$_2$CO$_2$), 2.49 (2 H, t, *J* 7.7, C**H**$_2$CH$_2$CO$_2$), 1.41 (9 H, s, C**Me**$_3$), 1.30 (3 H, t, *J* 7.1, CH$_2$**Me**); *m/z* (FAB) 819 (MH$^+$), 841 (MNa$^+$)

(Found: MNa^+, 841.3181. $C_{43}H_{51}{}^{35}ClN_4O_{10}$ requires MNa^+, 841.3191); COSY spectra exhibited a good correlation with the proposed structure.

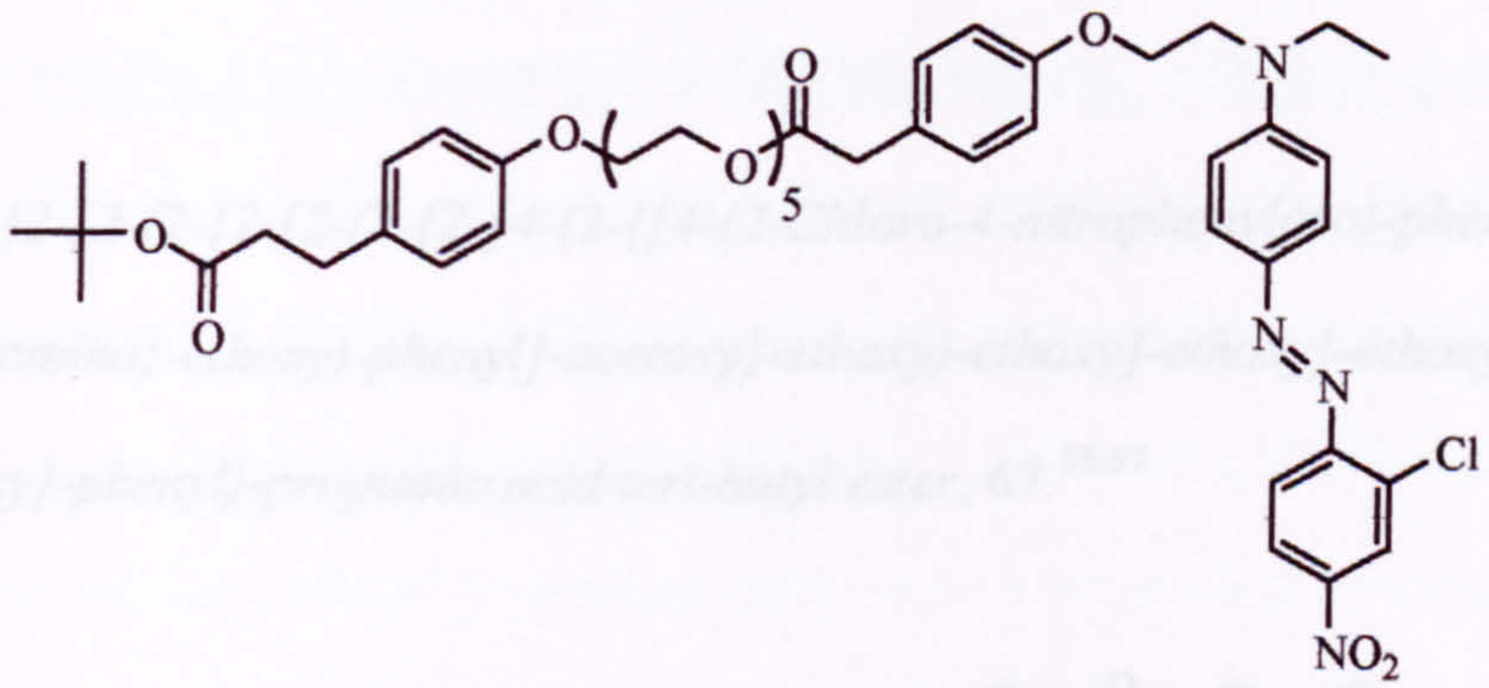
3-[4-(2-{2-[2-(2-{2-[4-(2-{[4-(2-Chloro-4-nitrophenylazo)-phenyl]-ethylamino}-ethoxy)-phenyl]-acetoxy}-ethoxy)-ethoxy]-ethoxy}-ethoxy)-phenyl]-propionic acid tert-butyl ester, **65**.[55-57]



To a solution of **48** (300 mg, 0.46 mmol), triphenylphosphine (179 mg, 0.68 mmol, 1.5 eq) and diethyl azodicarboxylate (120 mg, 0.68 mmol, 1.5 eq) in dichloromethane (5 ml) was added *tert*-butyl ester **62** (202 mg, 0.91 mmol, 2.0 eq). The reaction mixture was stirred at room temperature for 5 hours after which time TLC (methanol:ethyl acetate, 1:9) indicated that the starting material had been consumed. The dichloromethane was removed under reduced pressure and the product isolated by gradient flash column chromatography (ethyl acetate:hexane, 4:1; methanol:ethyl acetate, 1:9) to give **65** as a dark red oil, (204 mg, 0.24 mmol, 52%). ν_{max}(Nujol)/cm^{-1} 1733, 1601, 1513, 1266; δ_H(300 MHz; $CDCl_3$) 8.38 (1 H, d, *J* 2.4, ArC**H**), 8.14 (1 H, dd, *J* 8.9, 2.4, ArC**H**), 7.94 (2 H, d, J_{AB} 9.0, ArC**H**), 7.78 (1 H, d, *J* 8.9, ArC**H**), 7.19 (2 H, d, J_{AB} 8.7, ArC**H**), 7.09 (2 H, d, J_{AB} 8.7, ArC**H**), 6.86-6.79 (6 H, m, ArC**H**), 4.24 (2 H, t, *J* 5.3,

ArOC**H**$_2$CH$_2$), 4.17 (2 H, t, *J* 5.7, ArOC**H**$_2$CH$_2$), 4.09 (2 H, t, *J* 4.9, OCH$_2$C**H**$_2$O$_2$C), 3.87-3.56 (16 H, m, CH$_2$), 2.83 (2 H, t, *J* 7.7, CH$_2$C**H**$_2$CO$_2$), 2.49 (2 H, t, *J* 7.7, C**H**$_2$CH$_2$CO$_2$), 1.41 (9 H, s, C**Me**$_3$), 1.30 (3 H, t, *J* 7.1, CH$_2$**Me**); *m/z* (FAB) 863 (MH$^+$), 885 (MNa$^+$) (Found: MH$^+$, 863.3619. $C_{45}H_{55}{}^{35}ClN_4O_{11}$ requires MH$^+$, 863.3634); COSY spectra exhibited a good correlation with the proposed structure.

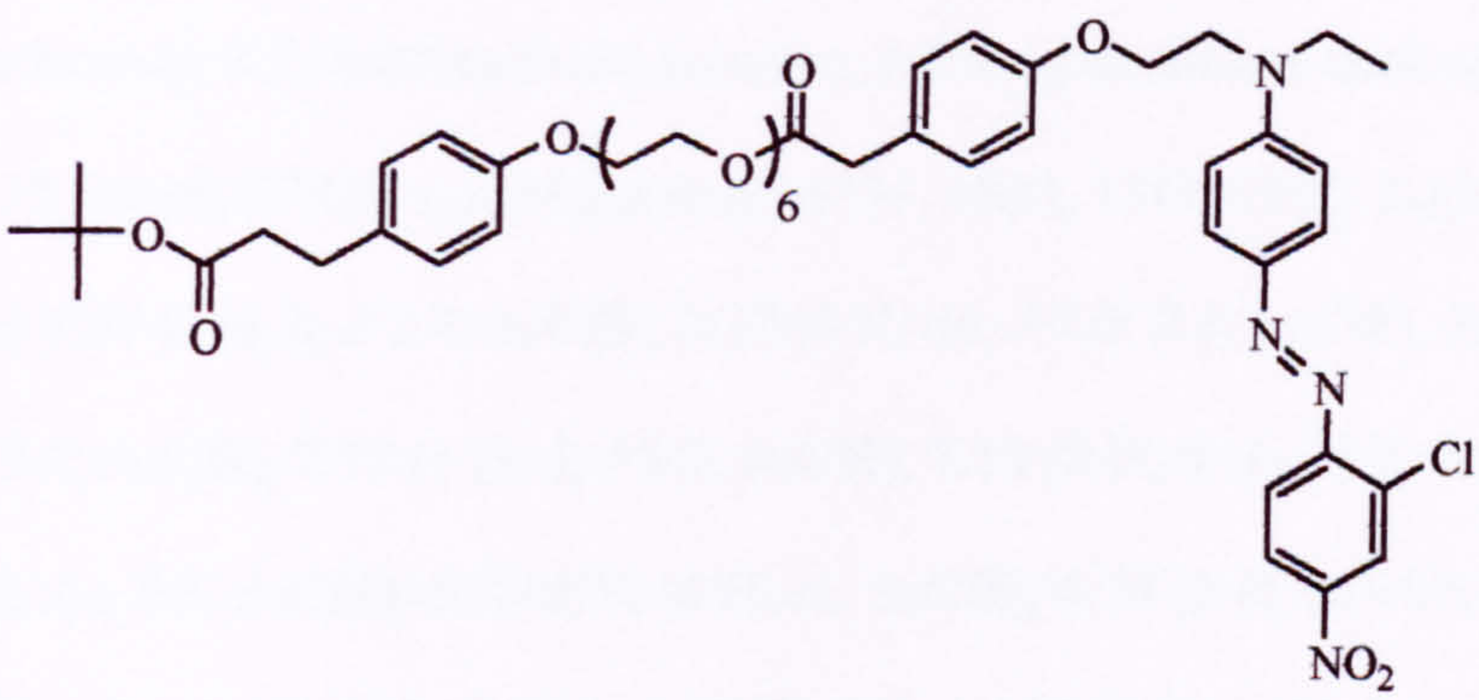
3-{4-[2-(2-{2-[2-(2-{2-[4-(2-{[4-(2-Chloro-4-nitrophenylazo)-phenyl]-ethylamino}-ethoxy)-phenyl]-acetoxy}-ethoxy)-ethoxy]-ethoxy}-ethoxy)-ethoxy]-phenyl}-propionic acid tert-butyl ester, **66**.[55-57]



To a solution of **49** (280 mg, 0.40 mmol), triphenylphosphine (108 mg, 0.60 mmol, 1.5 eq) and diethyl azodicarboxylate (104 mg, 0.60 mmol, 1.5 eq) in dichloromethane (5 ml) was added *tert*-butyl ester **62** (177 mg, 0.80 mmol, 2.0 eq). The reaction mixture was stirred at room temperature for 5 hours after which time TLC (methanol:ethyl acetate, 1:9) indicated that the starting material had been consumed. The dichloromethane was removed under reduced pressure and the product isolated by gradient flash column chromatography (ethyl

acetate:hexane, 4:1; methanol:ethyl acetate, 1:9) to give **66** as a dark red oil, (187 mg, 0.21 mmol, 53%). ν_{max}(Nujol)/cm^{-1} 1730, 1601, 1513, 1340; δ_H(300 MHz; $CDCl_3$) 8.37 (1 H, d, *J* 2.4, ArC**H**), 8.13 (1 H, dd, *J* 9.0, 2.4, ArC**H**), 7.94 (2 H, d, J_{AB} 9.2, ArC**H**), 7.77 (1 H, d, *J* 9.0, ArC**H**), 7.20 (2 H, d, J_{AB} 8.9, ArC**H**), 7.09 (2 H, d, J_{AB} 8.7, ArC**H**), 6.86-6.79 (6 H, m, ArC**H**), 4.23 (2 H, t, *J* 4.8, ArOC**H**$_2$CH$_2$), 4.17 (2 H, t, *J* 5.7, ArOC**H**$_2$CH$_2$), 4.09 (2 H, t, *J* 5.0, OCH$_2$C**H**$_2$O$_2$C), 3.87-3.81 (4 H, m, CH$_2$), 3.73-3.58 (18 H, m, C**H**$_2$), 2.83 (2 H, t, *J* 7.8, CH$_2$C**H**$_2$CO$_2$), 2.49 (2 H, t, *J* 7.8, C**H**$_2$CH$_2$CO$_2$), 1.41 (9 H, s, C**Me**$_3$), 1.29 (3 H, t, *J* 7.1, CH$_2$**Me**); *m/z* (FAB) 907 (MH$^+$), 929 (MNa$^+$) (Found: MH$^+$, 907.3904. $C_{47}H_{59}{}^{35}ClN_4O_{12}$ requires MH$^+$, 907.3896); COSY spectra exhibited a good correlation with the proposed structure.

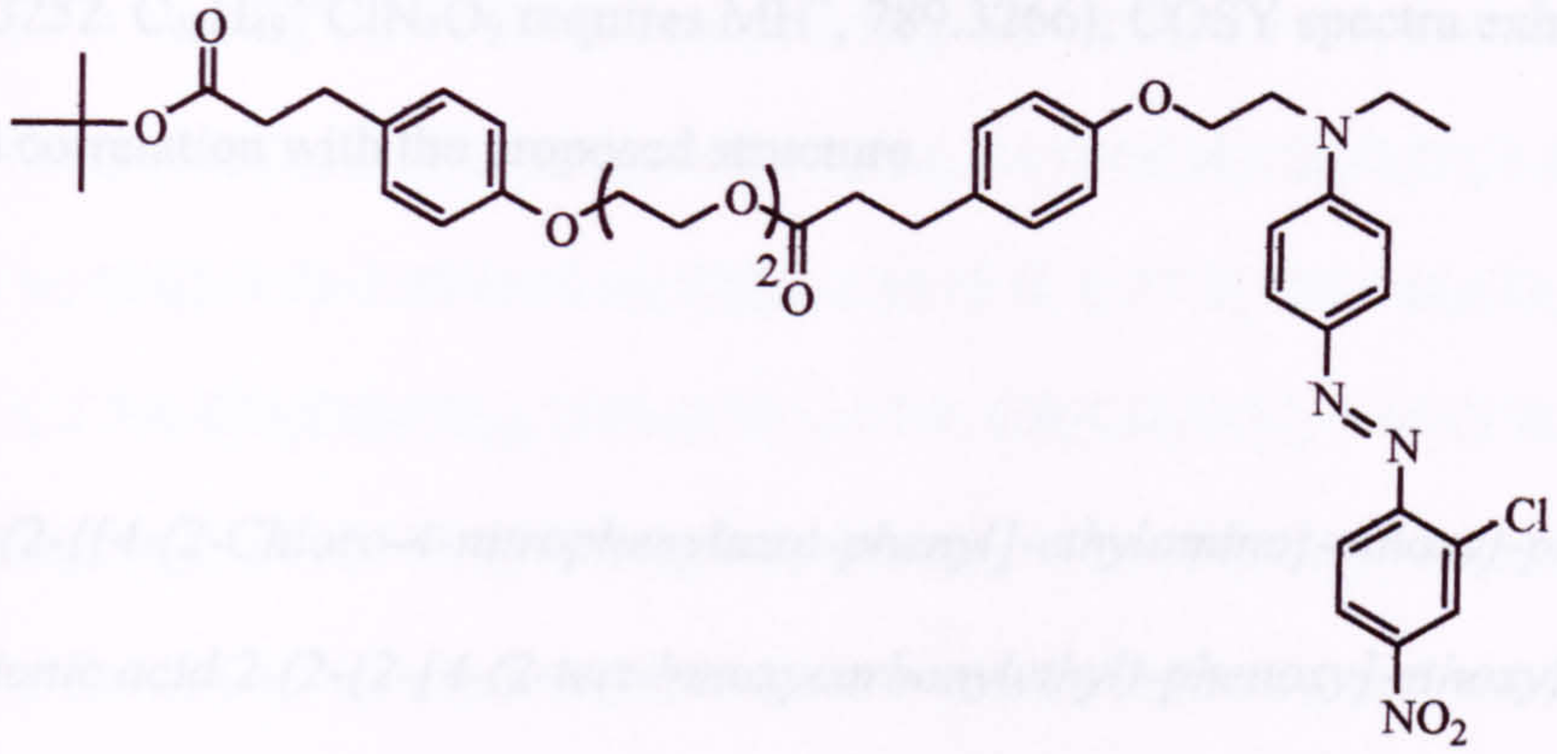
3-(4-{2-[2-(2-{2-[2-(2-{2-[4-(2-{[4-(2-Chloro-4-nitrophenylazo)-phenyl]-ethylamino}-ethoxy)-phenyl]-acetoxy}-ethoxy)-ethoxy]-ethoxy}-ethoxy)-ethoxy]-ethoxy}-phenyl)-propionic acid tert-butyl ester, **67**.[55-57]



To a solution of **50** (245 mg, 0.33 mmol), triphenylphosphine (129 mg, 0.49 mmol, 1.5 eq) and diethyl azodicarboxylate (86 mg, 0.49 mmol, 1.5 eq) in

dichloromethane (5 ml) was added *tert*-butyl ester **62** (146 mg, 0.66 mmol, 2.0 eq). The reaction mixture was stirred at room temperature for 5 hours after which time TLC (methanol:ethyl acetate, 1:9) indicated that the starting material had been consumed. The dichloromethane was removed under reduced pressure and the product isolated by gradient flash column chromatography (ethyl acetate:hexane, 4:1; methanol:ethyl acetate, 1:9) to give **67** as a dark red oil, (184 mg, 0.19 mmol, 58%). ν_{max}(Nujol)/cm^{-1} 1731, 1601, 1514, 1340; δ_H(300 MHz; $CDCl_3$) 8.37 (1 H, d, *J* 2.4, ArC**H**), 8.13 (1 H, dd, *J* 9.1, 2.4, ArC**H**), 7.94 (2 H, d, J_{AB} 9.2, ArC**H**), 7.77 (1 H, d, *J* 9.1, ArC**H**), 7.19 (2 H, d, J_{AB} 8.7, ArC**H**), 7.09 (2 H, d, J_{AB} 8.7, ArC**H**), 6.86-6.79 (6 H, m, ArC**H**), 4.23 (2 H, t, *J* 4.8, ArOC**H**$_2$CH$_2$), 4.17 (2 H, t, *J* 5.7, ArOC**H**$_2$CH$_2$), 4.09 (2 H, t, *J* 4.9, $OCH_2C\mathbf{H}_2O_2C$), 3.87-3.81 (4 H, m, CH_2), 3.73-3.55 (22 H, m, C**H**$_2$), 2.83 (2 H, t, *J* 7.8, $CH_2C\mathbf{H}_2CO_2$), 2.49 (2 H, t, *J* 7.8, C**H**$_2$CH$_2$CO$_2$), 1.41 (9 H, s, C**Me**$_3$), 1.29 (3 H, t, *J* 7.1, CH$_2$**Me**); *m/z* (FAB) 951 (MH^+), 973 (MNa^+) (Found: MH^+, 951.4167. $C_{49}H_{63}{}^{35}ClN_4O_{13}$ requires MH^+, 951.4158); COSY spectra exhibited a good correlation with the proposed structure.

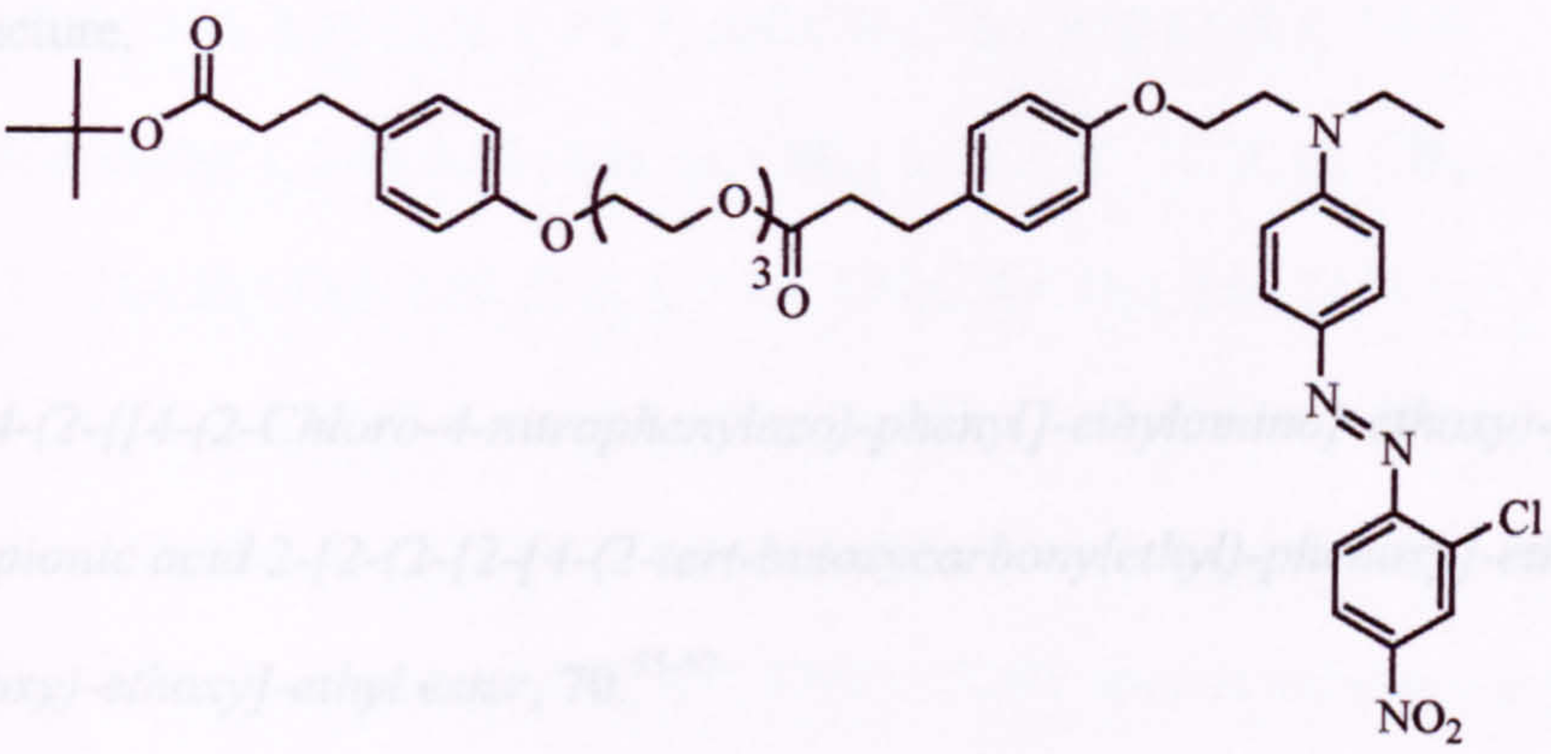
3-[4-(2-{[4-(2-Chloro-4-nitrophenylazo)-phenyl]-ethylamino}-ethoxy)-phenyl]-propionic acid 2-{2-[4-(2-tert-butoxycarbonylethyl)-phenoxy]-ethoxy}-ethyl ester, **68**.[55-57]



To a solution of **51** (178 mg, 0.30 mmol), triphenylphosphine (120 mg, 0.46 mmol, 1.5 eq) and diethyl azodicarboxylate (78 mg, 0.45 mmol, 1.5 eq) in dichloromethane (5 ml) was added *tert*-butyl ester **62** (135 mg, 0.61 mmol, 2.0 eq). The reaction mixture was stirred at room temperature for 5 hours after which time TLC (methanol:ethyl acetate, 1:9) indicated that the starting material had been consumed. The dichloromethane was removed under reduced pressure and the product isolated by gradient flash column chromatography (ethyl acetate:hexane, 4:1; methanol:ethyl acetate, 1:9) to give **68** as a dark red oil, (138 mg, 0.18 mmol, 57%). ν_{max}(Nujol)/cm^{-1} 1735, 1600, 1513, 1339; δ_H(300 MHz; $CDCl_3$) 8.37 (1 H, d, *J* 2.4, ArC**H**), 8.13 (1 H, dd, *J* 9.0, 2.4, ArC**H**), 7.94 (2 H, d, J_{AB} 9.4, ArC**H**), 7.77 (1 H, d, *J* 9.0, ArC**H**), 7.11 (2 H, d, J_{AB} 8.7, ArC**H**), 7.09 (2 H, d, J_{AB} 8.9, ArC**H**), 6.83-6.78 (6 H, m, ArC**H**), 4.24 (2 H, t, *J* 4.7, ArOC**H**$_2$CH$_2$), 4.16 (2 H, t, *J* 5.7, ArOC**H**$_2$CH$_2$), 4.08 (2 H, t, *J* 4.8, OCH$_2$C**H**$_2$O$_2$C), 3.83 (2 H, t, *J* 5.7, ArOCH$_2$C**H**$_2$), 3.81 (2 H, t, *J* 4.7, ArOCH$_2$C**H**$_2$), 3.74 (2 H, t, *J* 4.8, OC**H**$_2$CH$_2$O$_2$C), 3.61 (2 H, q, *J* 7.1, C**H**$_2$Me),

2.88 (2 H, t, *J* 7.8, $CH_2CH_2CO_2$), 2.84 (2 H, t, *J* 7.8, $CH_2CH_2CO_2$), 2.61 (2 H, t, *J* 7.8, $CH_2CH_2CH_2CO_2$), 2.49 (2 H, t, *J* 7.8, $CH_2CH_2CH_2CO_2$), 1.41 (9 H, s, CMe_3), 1.28 (3 H, t, *J* 7.1, CH_2Me); *m/z* (FAB) 789 (MH^+), 811 (MNa^+) (Found: MH^+, 789.3252. $C_{42}H_{49}{}^{35}ClN_4O_9$ requires MH^+, 789.3266); COSY spectra exhibited a good correlation with the proposed structure.

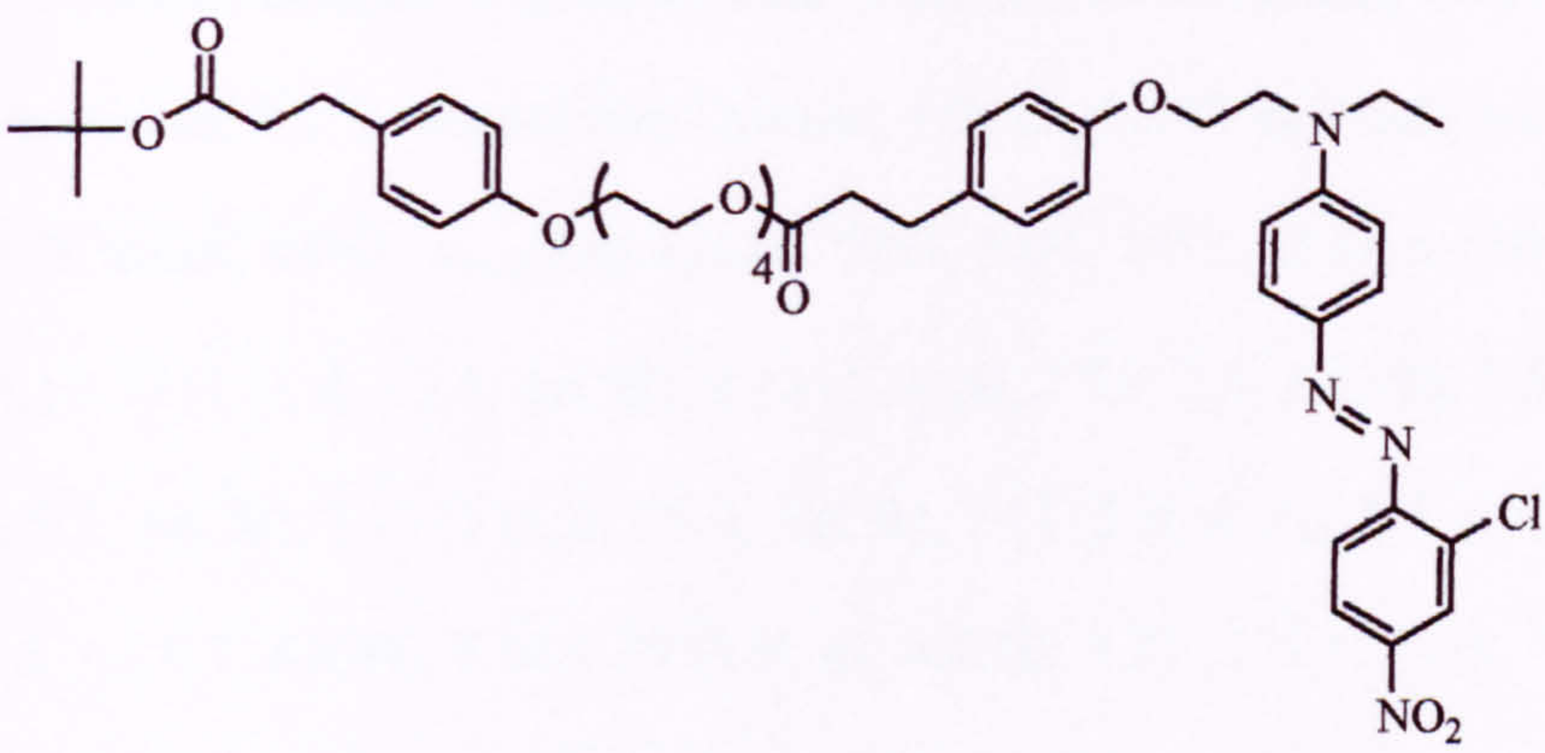
3-[4-(2-{[4-(2-Chloro-4-nitrophenylazo)-phenyl]-ethylamino}-ethoxy)-phenyl]-propionic acid 2-(2-{2-[4-(2-tert-butoxycarbonylethyl)-phenoxy]-ethoxy}-ethoxy)-ethyl ester, **69**.[55-57]



To a solution of **52** (767 mg, 1.22 mmol), triphenylphosphine (480 mg, 1.83 mmol, 1.5 eq) and diethyl azodicarboxylate (318 mg, 1.83 mmol, 1.5 eq) in dichloromethane (5 ml) was added *tert*-butyl ester **62** (542 mg, 2.44 mmol, 2.0 eq). The reaction mixture was stirred at room temperature for 5 hours after which time TLC (methanol:ethyl acetate, 1:9) indicated that the starting material had been consumed. The dichloromethane was under reduced pressure and the product isolated by gradient flash column chromatography (ethyl acetate:hexane, 4:1; methanol:ethyl acetate, 1:9) to give **69** as a dark red oil, (386 mg, 0.46

mmol, 38%). ν_{max}(Nujol)/cm^{-1} 1736, 1600, 1512, 1339; δ_H(300 MHz; $CDCl_3$) 8.37 (1 H, d, *J* 2.5, ArC**H**), 8.13 (1 H, dd, *J* 9.0, 2.5, ArC**H**), 7.93 (2 H, d, J_{AB} 9.4, ArC**H**), 7.77 (1 H, d, *J* 9.0, ArC**H**), 7.11 (2 H, d, J_{AB} 8.9, ArC**H**), 7.09 (2 H, d, J_{AB} 8.9, ArC**H**), 6.83-6.79 (6 H, m, ArC**H**), 4.24 (2 H, t, *J* 4.7, ArOC**H**$_2$CH$_2$), 4.16 (2 H, t, *J* 5.7, ArOC**H**$_2$CH$_2$), 4.09 (2 H, t, *J* 4.9, OCH$_2$C**H**$_2$O$_2$C), 3.86-3.81 (4 H, m, CH$_2$), 3.75-3.58 (8 H, m, CH$_2$), 2.88 (2 H, t, *J* 7.8, CH$_2$C**H**$_2$CO$_2$), 2.83 (2 H, t, *J* 7.8, CH$_2$C**H**$_2$CO$_2$), 2.61 (2 H, t, *J* 7.8, C**H**$_2$CH$_2$CO$_2$), 2.49 (2 H, t, *J* 7.8, C**H**$_2$CH$_2$CO$_2$), 1.41 (9 H, s, C**Me**$_3$), 1.29 (3 H, t, *J* 7.0, CH$_2$**Me**); *m/z* (FAB) 833 (MH$^+$), 855 (MNa$^+$) (Found: MH$^+$, 833.3507. $C_{44}H_{53}{}^{35}ClN_4O_{10}$ requires MH$^+$, 833.3528); COSY spectra exhibited a good correlation with the proposed structure.

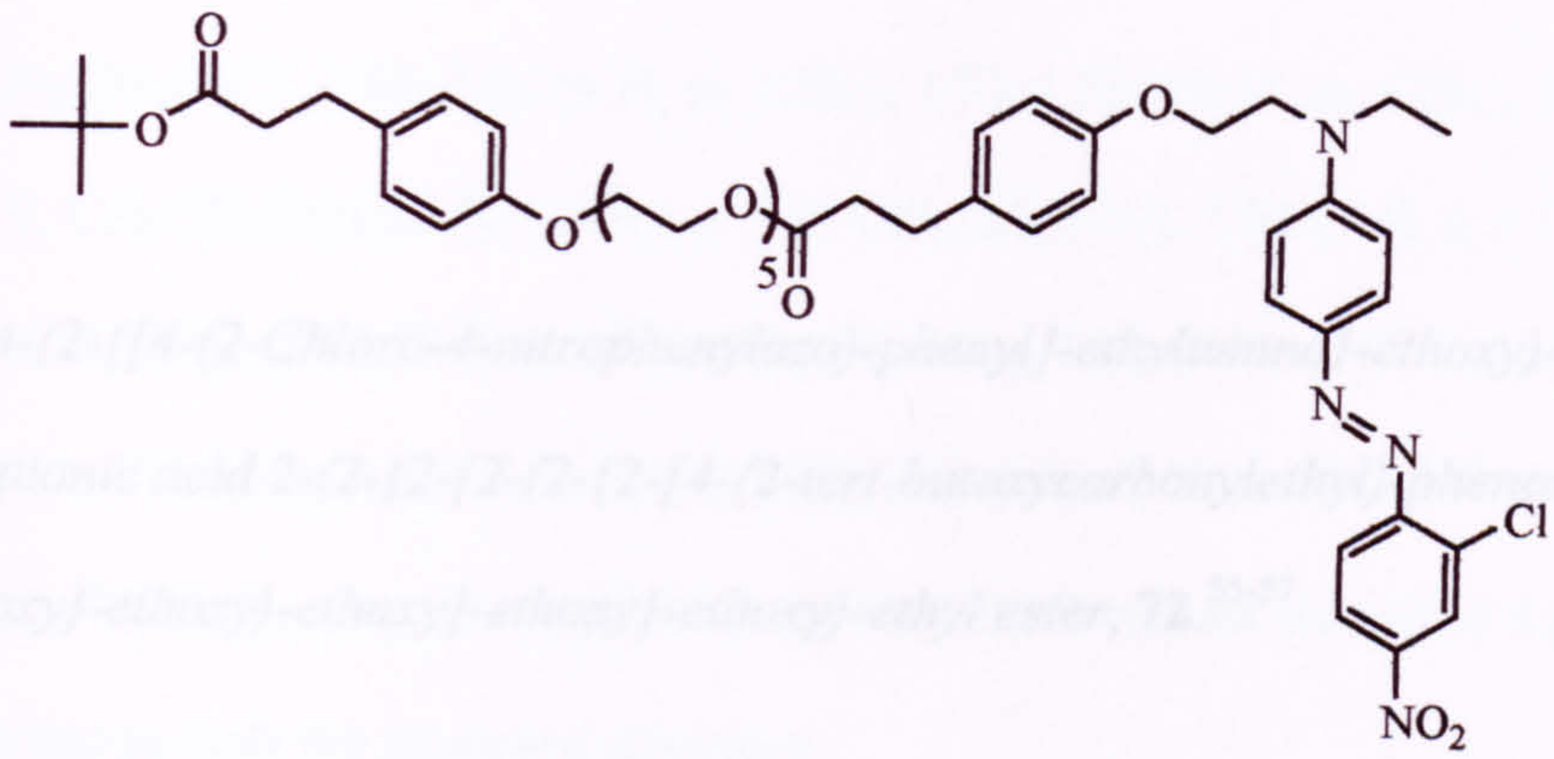
3-[4-(2-{[4-(2-Chloro-4-nitrophenylazo)-phenyl]-ethylamino}-ethoxy)-phenyl]-propionic acid 2-[2-(2-{2-[4-(2-tert-butoxycarbonylethyl)-phenoxy]-ethoxy}-ethoxy)-ethoxy]-ethyl ester, **70**.[55-57]



To a solution of **53** (470 mg, 0.70 mmol), triphenylphosphine (275 mg, 1.05 mmol, 1.5 eq) and diethyl azodicarboxylate (183 mg, 1.05 mmol, 1.5 eq) in

dichloromethane (5 ml) was added *tert*-butyl ester **62** (311 mg, 1.40 mmol, 2.0 eq). The reaction mixture was stirred at room temperature for 5 hours after which time TLC (methanol:ethyl acetate, 1:9) indicated that the starting material had been consumed. The dichloromethane was removed under reduced pressure and the product isolated by gradient flash column chromatography (ethyl acetate:hexane, 4:1; methanol:ethyl acetate, 1:9) to give **70** as a dark red oil, (291 mg, 0.33 mmol, 47%). ν_{max}(Nujol)/cm^{-1} 1728, 1600, 1513, 1340; δ_H(300 MHz; $CDCl_3$) 8.34 (1 H, d, *J* 2.5, ArC**H**), 8.10 (1 H, dd, *J* 8.9, 2.5, ArC**H**), 7.92 (2 H, d, J_{AB} 9.2, ArC**H**), 7.75 (1 H, d, *J* 8.9, ArC**H**), 7.11 (2 H, d, J_{AB} 8.7, ArC**H**), 7.09 (2 H, d, J_{AB} 8.7, ArC**H**), 6.84-6.77 (6 H, m, ArC**H**), 4.21 (2 H, t, *J* 4.8, ArOC**H**$_2$CH$_2$), 4.15 (2 H, t, *J* 5.7, ArOC**H**$_2$CH$_2$), 4.08 (2 H, t, *J* 4.9, OCH$_2$C**H**$_2$O$_2$C), 3.85-3.80 (4 H, m, C**H**$_2$), 3.73-3.57 (12 H, m, C**H**$_2$), 2.88 (2 H, t, *J* 7.7, CH$_2$C**H**$_2$CO$_2$), 2.83 (2 H, t, *J* 7.7, CH$_2$C**H**$_2$CO$_2$), 2.61 (2 H, t, *J* 7.7, C**H**$_2$CH$_2$CO$_2$), 2.49 (2 H, t, *J* 7.7, C**H**$_2$CH$_2$CO$_2$), 1.41 (9 H, s, C**Me**$_3$), 1.28 (3 H, t, *J* 7.1, CH$_2$**Me**); *m/z* (FAB) 877 (MH$^+$), 899 (MNa$^+$) (Found: MH$^+$, 877.3802. $C_{46}H_{57}{}^{35}ClN_4O_{11}$ requires MH$^+$, 877.3790); COSY spectra exhibited a good correlation with the proposed structure.

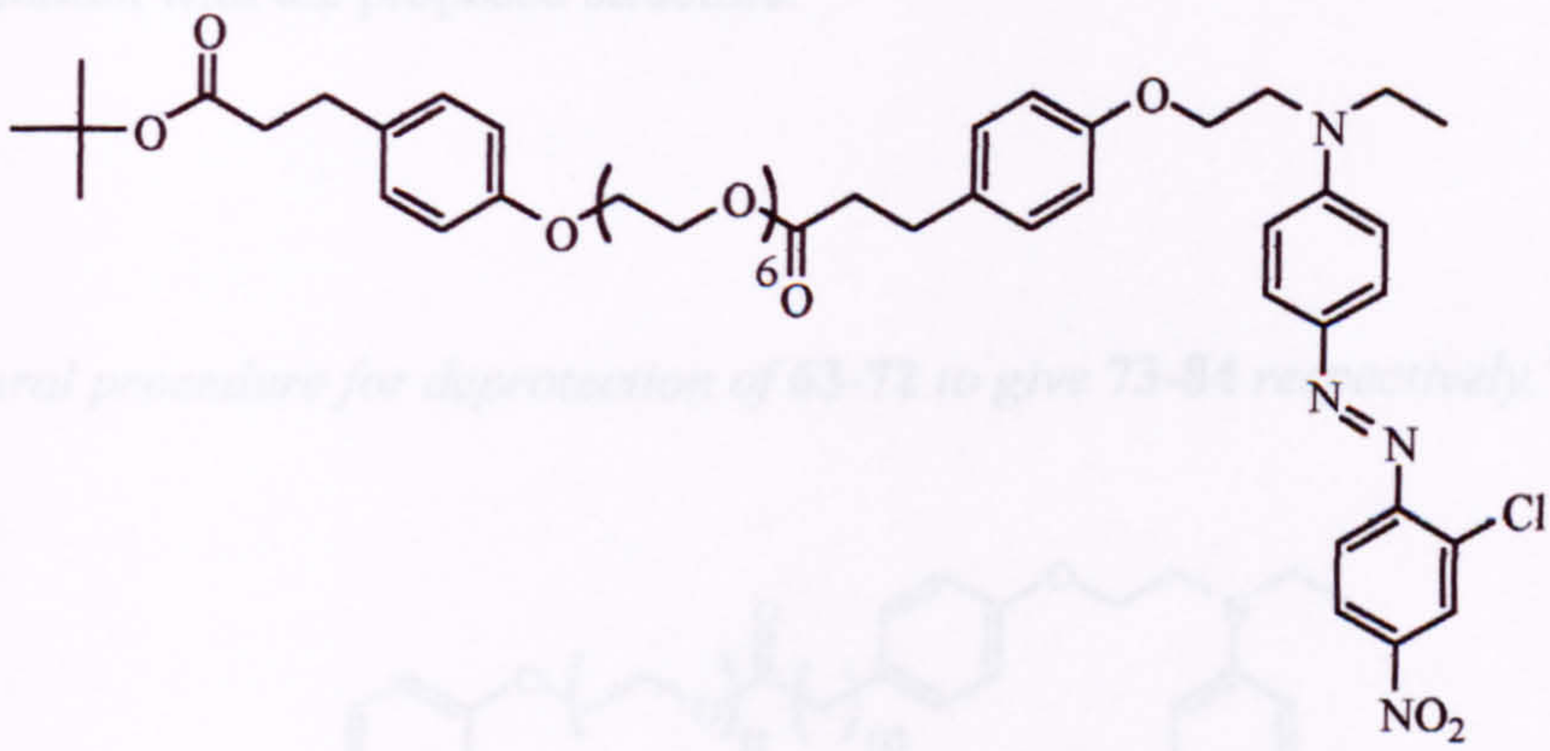
3-[4-(2-{[4-(2-Chloro-4-nitrophenylazo)-phenyl]-ethylamino}-ethoxy)-phenyl]-propionic acid 2-{2-[2-(2-{2-[4-(2-tert-butoxycarbonylethyl)-phenoxy]-ethoxy}-ethoxy)-ethoxy]-ethoxy}-ethyl ester, **71**.[55-57]



To a solution of **54** (420 mg, 0.59 mmol), triphenylphosphine (231 mg, 0.88 mmol, 1.5 eq) and diethyl azodicarboxylate (154 mg, 0.89 mmol, 1.5 eq) in dichloromethane (5 ml) was added *tert*-butyl ester **62** (260 mg, 1.17 mmol, 2.0 eq). The reaction mixture was stirred at room temperature for 5 hours after which time TLC (methanol:ethyl acetate, 1:9) indicated that the starting material had been consumed. The dichloromethane was removed under reduced pressure and the product isolated by gradient flash column chromatography (ethyl acetate:hexane, 4:1; methanol:ethyl acetate, 1:9) to give **71** as a dark red oil, (235 mg, 0.26 mmol, 44%). ν_{max}(Nujol)/cm^{-1} 1727, 1601, 1513, 1340; δ_H(300 MHz; $CDCl_3$) 8.37 (1 H, d, *J* 2.5, ArC**H**), 8.13 (1 H, dd, *J* 9.0, 2.5, ArC**H**), 7.94 (2 H, d, J_{AB} 9.2, ArC**H**), 7.77 (1 H, d, *J* 9.0, ArC**H**), 7.11 (2 H, d, J_{AB} 8.7, ArC**H**), 7.09 (2 H, d, J_{AB} 8.7, ArC**H**), 6.84-6.79 (6 H, m, ArC**H**), 4.21 (2 H, t, *J* 4.8, ArOC**H**$_2$CH$_2$), 4.16 (2 H, t, *J* 5.7, ArOC**H**$_2$CH$_2$), 4.09 (2 H, t, *J* 4.9, OCH$_2$C**H**$_2$O$_2$C), 3.86-3.81 (4 H, m, C**H**$_2$), 3.73-3.56 (16 H, m, C**H**$_2$), 2.89 (2 H, t, *J* 7.7, CH$_2$C**H**$_2$CO$_2$), 2.83 (2 H, t, *J* 7.7, CH$_2$C**H**$_2$CO$_2$), 2.61 (2 H, t, *J* 7.7,

C**H**$_2$CH$_2$CO$_2$), 2.49 (2 H, t, *J* 7.7, C**H**$_2$CH$_2$CO$_2$), 1.41 (9 H, s, C**Me**$_3$), 1.29 (3 H, t, *J* 7.1, CH$_2$**Me**); *m/z* (FAB) 921 (MH^+), 943 (MNa^+) (Found: MH^+, 921.4048. $C_{48}H_{61}{}^{35}ClN_4O_{12}$ requires MH^+, 921.4053); COSY spectra exhibited a good correlation with the proposed structure.

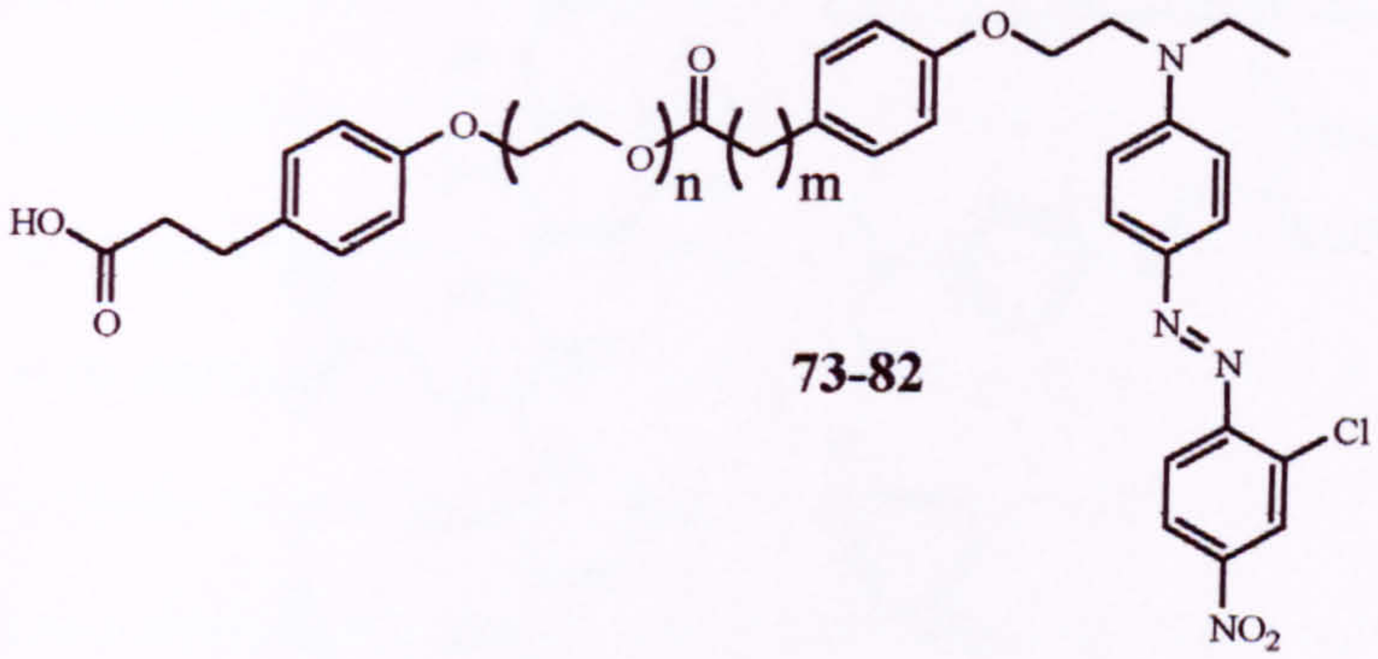
3-[4-(2-{[4-(2-Chloro-4-nitrophenylazo)-phenyl]-ethylamino}-ethoxy)-phenyl]-propionic acid 2-(2-{2-[2-(2-{2-[4-(2-tert-butoxycarbonylethyl)-phenoxy]-ethoxy}-ethoxy)-ethoxy]-ethoxy}-ethoxy)-ethyl ester, **72**.[55-57]



To a solution of **55** (767 mg, 1.01 mmol), triphenylphosphine (397 mg, 1.51 mmol, 1.5 eq) and diethyl azodicarboxylate (264 mg, 1.51 mmol, 1.5 eq) in dichloromethane (5 ml) was added *tert*-butyl ester **62** (448 mg, 2.02 mmol, 2.0 eq). The reaction mixture was stirred at room temperature for 5 hours after which time TLC (methanol:ethyl acetate, 1:9) indicated that the starting material had been consumed. The dichloromethane was removed under reduced pressure and the product isolated by gradient flash column chromatography (ethyl acetate:hexane, 4:1; methanol:ethyl acetate, 1:9) to give **72** as a dark red oil, (549 mg, 0.57 mmol, 56%). ν_{max}(Nujol)/cm^{-1} 1727, 1601, 1513, 1340; δ_H(300 MHz;

$CDCl_3$) 8.37 (1 H, d, *J* 2.5, ArC**H**), 8.13 (1 H, dd, *J* 9.0, 2.5, ArC**H**), 7.94 (2 H, d, J_{AB} 9.2, ArC**H**), 7.77 (1 H, d, *J* 9.0, ArC**H**), 7.11 (2 H, d, J_{AB} 8.7, ArC**H**), 7.09 (2 H, d, J_{AB} 8.9, ArC**H**), 6.83-6.79 (6 H, m, ArC**H**), 4.21 (2 H, t, *J* 4.8, $ArOC\mathbf{H}_2CH_2$), 4.16 (2 H, t, *J* 5.7, $ArOC\mathbf{H}_2CH_2$), 4.09 (2 H, t, *J* 4.9, $OCH_2C\mathbf{H}_2O_2C$), 3.86-3.81 (4 H, m, $C\mathbf{H}_2$), 3.73-3.56 (20 H, m, $C\mathbf{H}_2$), 2.89 (2 H, t, *J* 7.7, $CH_2C\mathbf{H}_2CO_2$), 2.83 (2 H, t, *J* 7.7, $CH_2C\mathbf{H}_2CO_2$), 2.61 (2 H, t, *J* 7.7, $C\mathbf{H}_2CH_2CO_2$), 2.49 (2 H, t, *J* 7.7, $C\mathbf{H}_2CH_2CO_2$), 1.31 (9 H, s, $C\mathbf{Me}_3$), 1.29 (3 H, t, *J* 7.1, $CH_2\mathbf{Me}$); *m/z* (FAB) 965 (MH^+), 987 (MNa^+) (Found: MH^+, 965.4323. $C_{50}H_{65}{}^{35}ClN_4O_{13}$ requires MH^+, 965.4315); COSY spectra exhibited a good correlation with the proposed structure.

General procedure for deprotection of **63-72** *to give* **73-84** *respectively.*[79]



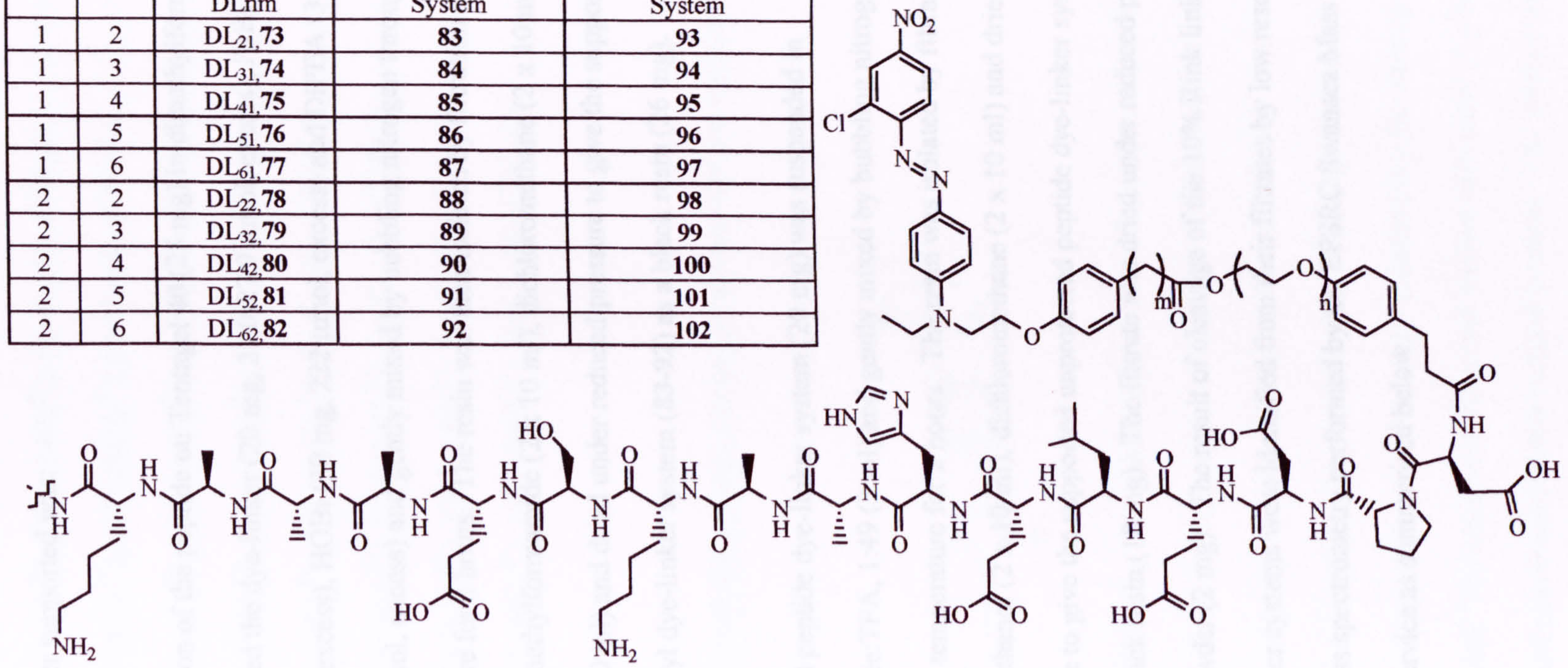
73-82

To a solution of TIS:TFA, 1:49 (10 ml) was added a solution of **63** (200 mg, 258 μmol) in dichloromethane (1.5 ml). The reaction mixture was stirred at room temperature for 10 minutes after which time TLC (ethyl acetate:hexane, 3:2) indicated that the starting material had been consumed. The reaction mixture was concentrated under reduced pressure and the product isolated *via* flash column chromatography to give **73** as a red oil (158 mg, 220 μmol, 85%). The

acid derivative was then coupled directly to the supported protected peptide **40** as described below.

Individual Attachment of the Dye-Linkers **73-82** *to the supported 16 residue peptide* **40**.

m	n	Dye-Linker DLnm	Protected Peptidic System	Deprotected Peptidic System
1	2	DL_{21},**73**	**83**	**93**
1	3	DL_{31},**74**	**84**	**94**
1	4	DL_{41},**75**	**85**	**95**
1	5	DL_{51},**76**	**86**	**96**
1	6	DL_{61},**77**	**87**	**97**
2	2	DL_{22},**78**	**88**	**98**
2	3	DL_{32},**79**	**89**	**99**
2	4	DL_{42},**80**	**90**	**100**
2	5	DL_{52},**81**	**91**	**101**
2	6	DL_{62},**82**	**92**	**102**



General preparation for attaching the ten dye-linkers to the terminal amino group of the peptide, followed by deprotection and partial cleavage of these systems from the polymer supported was:

a) To a suspension of the peptide on Tentagel **40** (25 mg) in dimethylformamide (1 ml) were added the dye-linker (20 mg, 21.0-27.9 μmol, excess), PyBOP (30 mg, 57.7 μmol, excess), HOBt (30 mg, 222 μmol, excess) and DIPEA (3 drops, ~30 mg, 233 μmol, excess) and gently mixed by bubbling nitrogen through at room temperature for 3 hours. The resin was then isolated by filtration and washed with dimethylformamide (2 x 10 ml), dichloromethane (2 x 10 ml), methanol (2 x 10 ml) and dried under reduced pressure to give the supported protected peptidyl dye-linker system (**83-92**) as a black resin (26 mg).

b) The protected peptide dye-linker system (20 mg) was suspended in triisopropylamine:TFA, 1:49 (3 ml) and gently mixed by bubbling nitrogen through at room temperature for 2 hours. The resin was isolated by filtration, washed with methanol (2 x 10 ml), dichloromethane (2 x 10 ml) and dried under reduced pressure to give the supported unprotected peptide dye-linker system (**93-102**) as a black resin (18 mg). The filtrate was dried under reduced pressure to give a red powder (2 mg). The result of cleavage of the 10% Rink linker. The peptide dye-linker systems were identified from their filtrates by low resolution electrospray mass spectrometry performed by the EPSRC Swansea Mass Spectrometry Service as summarised below.

93; *m/z* (ES) 795 (MH_3^{3+}), 1191 (MH_2^{2+}), for $C_{107}H_{150}ClN_{25}O_{35}$.

94; *m/z* (ES) 809 (MH_3^{3+}), 1214 (MH_2^{2+}), for $C_{109}H_{154}ClN_{25}O_{36}$.

95; *m/z* (ES) 824 (MH_3^{3+}), 1236 (MH_2^{2+}), for $C_{111}H_{158}ClN_{25}O_{37}$.

96; *m/z* (ES) 839 (MH_3^{3+}), 1258 (MH_2^{2+}), for $C_{113}H_{162}ClN_{25}O_{38}$.

97; *m/z* (ES) 854 (MH_3^{3+}), 1280 (MH_2^{2+}), for $C_{115}H_{166}ClN_{25}O_{39}$.

98; *m/z* (ES) 780 (MH_3^{3+}), 1199 (MH_2^{2+}), for $C_{108}H_{152}ClN_{25}O_{35}$.

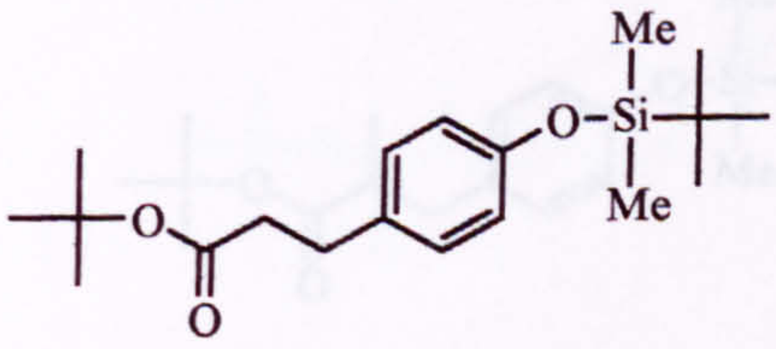
99; *m/z* (ES) 814 (MH_3^{3+}), 1221 (MH_2^{2+}), for $C_{110}H_{156}ClN_{25}O_{36}$.

100; *m/z* (ES) 829 (MH_3^{3+}), 1243 (MH_2^{2+}), for $C_{112}H_{160}ClN_{25}O_{37}$.

101; *m/z* (ES) 844 (MH_3^{3+}), 1265 (MH_2^{2+}), for $C_{114}H_{164}ClN_{25}O_{38}$.

102; *m/z* (ES) 858 (MH_3^{3+}), 1287 (MH_2^{2+}), for $C_{116}H_{168}ClN_{25}O_{39}$.

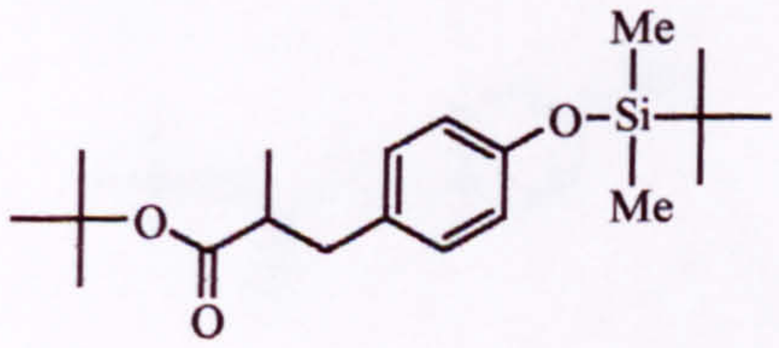
3-[4-(tert-Butyldimethylsilanyloxy)-phenyl]-propionic acid tert-butyl ester, **104**.[61]



To a solution of **62** (8.0 g, 36.0 mmol) in dichloromethane (100 ml) was added dimethylaminopyridine (1-2 crystals), triethylamine (7.80 ml, 54.1 mmol, 1.5 eq) and *tert*-butyldimethylsilyl chloride (5.95 g, 39.6 mmol, 1.1 eq). The reaction was stirred at room temperature for 8 hours after which time TLC (ethyl acetate:hexane, 1:9, and PMA dip to visualise) indicated that the starting material had been consumed. The reaction mixture was washed with water (3 x 20 ml), dried ($MgSO_4$) and concentrated under reduced pressure. The product was

isolated with flash column chromatography (ethyl acetate:hexane, 1:9) to give **104** as a clear colourless oil (9.60 g, 28.2 mmol, 79%). ν_{max}(Nujol)/cm^{-1} 1731, 1600, 1511; δ_H(300 MHz; $CDCl_3$) 7.06 (2 H, d, J_{AB} 8.5, ArC**H**), 6.77 (2 H, d, J_{AB} 8.5, ArC**H**), 2.86 (2 H, t, *J* 7.7, $ArCH_2C\mathbf{H}_2$), 2.52 (2 H, t, *J* 7.7, $ArC\mathbf{H}_2CH_2$), 1.43 (9 H, s, C**Me**$_3$), 1.00 (9 H, s, SiC*i***Me**$_2$), 0.20 (6 H, s, Si**Me**); δ_C(75 MHz; $CDCl_3$) 172.7 (C), 154.3 (C), 133.8 (C), 129.6 (2 CH), 120.3 (2 CH), 80.6 (C), 37.7 (CH_2), 30.8 (CH_2), 28.5 (CH_3), 26.1 (CH_3), 18.6 (C), -4.0 (CH_3); *m/z* (EI) 336 (M^+); (CI) 354 (MNH_4^+); (Found MNH_4^+, 354.2461. $C_{19}H_{32}O_3Si$ requires MNH_4^+, 354.2464); COSY spectra exhibited a good correlation with the proposed structure.

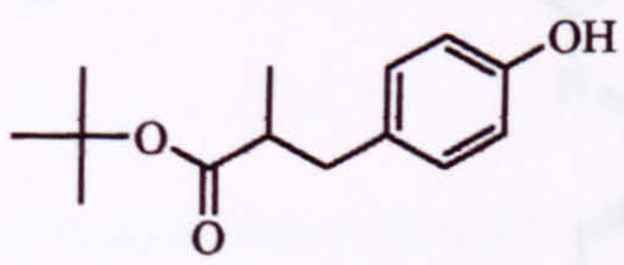
3-[4-(tert-Butyldimethylsilanyloxy)-phenyl]-2-methylpropionic acid tert-butyl ester, **105**.[99]



A solution of **104** (4.5 g, 13.4 mmol) in tetrahydrofuran (25 ml) was cooled to –78°C and stirred for 30 minutes. To this methyl iodide (8.2 ml, 132.4 mmol, 10 eq), HMPA (16.2 ml, 132.4 mmol, 10 eq) and LDA [2M solution in THF], (7.3 ml, 14.6 mmol, 1.1 eq) were added and stirred at –78°C for 6 hours. The reaction mixture was then stirred at room temperature overnight after which time TLC (diethyl ether:hexane, 1:9) indicated that the starting material had been consumed. The excess reagents and solvent were carefully removed under

reduced pressure and the product isolated *via* flash column chromatography (ethyl acetate:hexane, 1:9) to give **105** as a clear colourless oil (2.8 g, 8.0 mmol, 60%). ν_{max}(Nujol)/cm^{-1} 1729, 1600, 1510; δ_H(300 MHz; $CDCl_3$) 7.04 (2 H, d, J_{AB} 8.4, ArC**H**), 6.76 (2 H, d, J_{AB} 8.4, ArC**H**), 2.94-2.83 (1 H, m, $ArCH_2C$**H**), 2.64-2.49 (2 H, m, Ar**CH$_2$**CH), 1.39 (9 H, s, C**Me$_3$**), 1.12 (3 H, d, *J* 6.6, CH**Me**), 1.00 (9 H, s, SiC**Me$_3$**), 0.20 (6 H, s, Si**Me$_2$**); δ_C(75 MHz; $CDCl_3$) 172.7 (C), 154.3 (C), 133.8 (C), 129.6 (2 CH), 120.3 (2 CH), 80.6 (C), 42.9 (CH), 38.0 (CH_2), 28.5 (CH_3), 26.1 (CH_3), 16.5 (CH_3), 18.6 (C), -4.0 (CH_3); *m/z* (EI) 350 (M^+); (CI) 368 (MNH_4^+); (Found: MNH_4^+, 368.2622. $C_{20}H_{34}O_3Si$ requires MNH_4^+, 368.2621). COSY spectra exhibited a good correlation with the proposed structure.

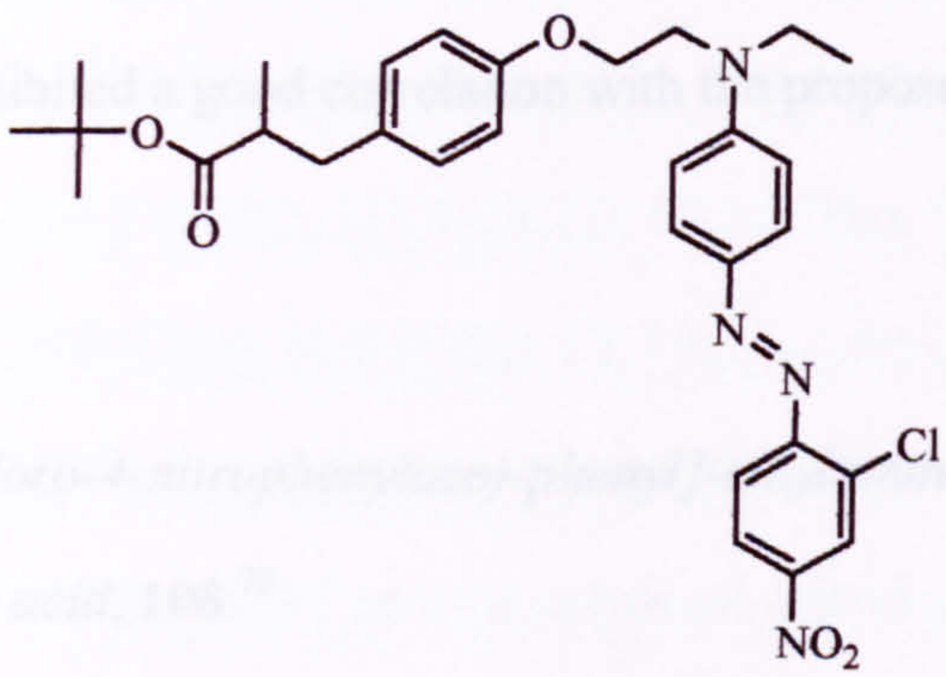
3-(4-Hydroxyphenyl)-2-methylpropionic acid tert-butyl ester, **106**.[61]



A solution of **105** (1.5 g, 4.29 mmol) in tetrahydrofuran (20 ml) was cooled to –10°C to which TBAF (1.34 g, 4.24 mmol) was quickly added. The reaction mixture was stirred at room temperature for 20 minutes after which time TLC (ethyl acetate:hexane, 1:4, and PMA dip to visualise) indicated that the starting material had been consumed. The reaction mixture was diluted with diethyl ether (40 ml), washed with water (3 x 20 ml), dried ($MgSO_4$) and concentrated under reduced pressure. The product was isolated *via* flash column chromatography (ethyl acetate:hexane, 1:4) to give **106** as a clear colourless oil

(830 mg, 3.52 mmol, 82%). ν_{max}(Nujol)/cm^{-1} 3392, 1728, 1600, 1516; δ_H(300 MHz; $CDCl_3$) 7.00 (2 H, d, J_{AB} 8.5, ArC**H**), 6.72 (2 H, d, J_{AB} 8.5, ArC**H**), 6.53 (1 H, s, ArO**H**), 2.89-2.80 (1 H, m, $ArCH_2C$**H**), 2.64-2.49 (2 H, m, Ar**C**$\mathbf{H}_2$CH), 1.38 (9 H, s, C**Me**$_3$), 1.11 (3 H, d, *J* 6.6, CH**Me**); δ_C(75 MHz; $CDCl_3$) 177.0 (C), 154.3 (C), 132.6 (C), 131.5 (2 CH), 115.7 (2 CH), 81.0 (C), 43.2 (CH), 39.4 (CH_2), 28.5 (CH_3), 17.4 (CH_3); *m/z* (EI) 236 (M^+); (CI) 254 (MNH_4^+); (Found MNH_4^+, 254.1752. $C_{14}H_{20}O_3$ requires MNH_4^+, 354.1756); COSY spectra exhibited a good correlation with the proposed structure.

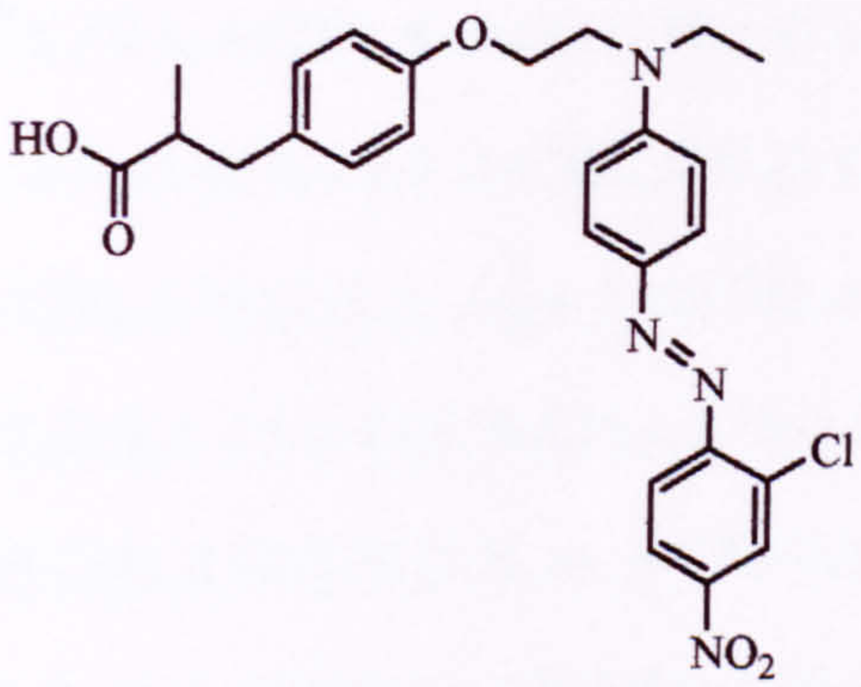
3-[4-(2-{[4-(2-Chloro-4-nitrophenylazo)-phenyl]-ethylamino}-ethoxy)-phenyl]-2-methylpropionic acid tert-butyl ester, **107**.[55-57]



To a solution of **106** (660 mg, 2.80 mmol) in dichloromethane (10 ml) was added triphenylphosphine (734 mg, 2.80 mmol), diethyl azodicarboxylate (0.44 ml, 2.80 mmol) and disperse red (974 mg, 2.80 mmol). The reaction mixture was stirred at room temperature for 3 hours after which time TLC (ethyl acetate:hexane, 1:4) indicated that the starting material had been consumed. The dichloromethane was removed under reduced pressure and the product isolated

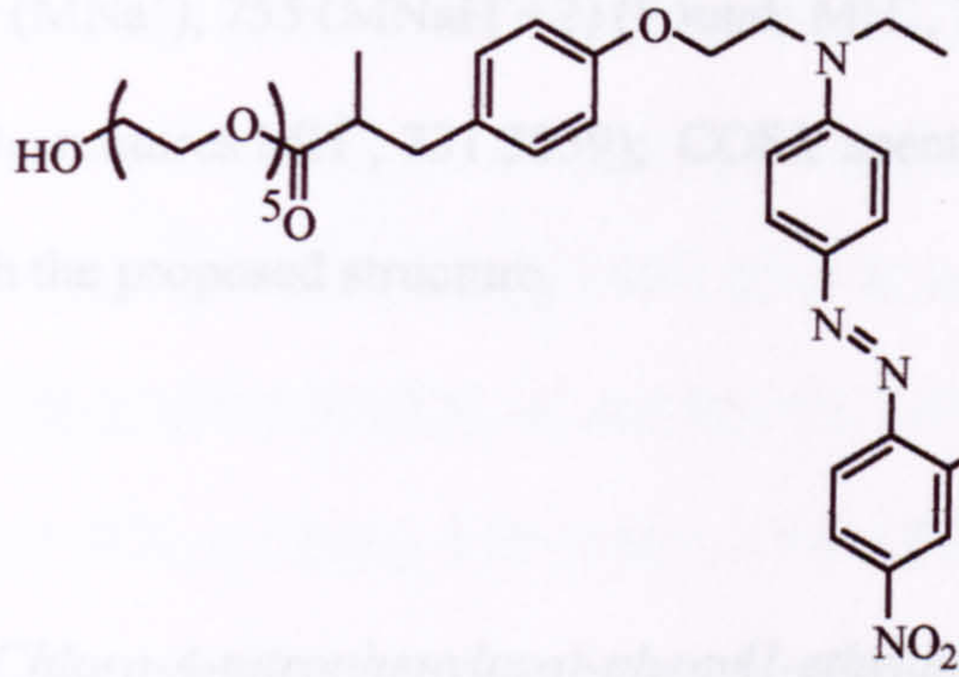
via flash column chromatograph to give **107** as a dark red oil (514 mg, 0.91 mmol, 33%). ν_{max}(Nujol)/cm^{-1}, 1724, 1601, 1514, 1337; δ_H(300 MHz; $CDCl_3$) 8.35 (1 H, d, *J* 2.5, ArC**H**), 8.11 (1 H, dd, *J* 8.9, 2.5, ArC**H**), 7.92 (2 H, d, J_{AB} 9.0, ArC**H**), 7.75 (1 H, d, *J* 8.9, ArC**H**), 7.08 (2 H, d, J_{AB} 9.0, ArC**H**), 6.79 (4 H, d, J_{AB} 9.0, ArC**H**), 4.16 (2 H, t, *J* 5.8, ArOC**H**$_2$CH$_2$), 3.84 (2 H, t, *J* 5.8, ArOCH$_2$C**H**$_2$), 3.61 (2 H, q, *J* 7.1, C**H**$_2$Me), 2.94-2.80 (1 H, m, ArCH$_2$C**H**), 2.63-2.47 (2 H, m, ArC**H**$_2$CH), 1.41 (9 H, s, C**Me**$_3$), 1.28 (3 H, t, *J* 7.1, CH$_2$**Me**), 1.09 (3 H, d, *J* 6.6, CH**Me**); δ_C(75 MHz; $CDCl_3$) 176.0 (C), 157.3 (C), 153.5 (C), 152.2 (C), 147.5 (C), 144.7 (C), 134.3 (C), 132.8 (C), 130.5 (2 CH), 127.4 (CH), 126.4 (CH), 123.0 (CH), 118.4 (2 CH), 114.6 (2 CH), 111.9 (2 CH), 81.5 (C), 65.7 (CH$_2$), 50.4 (CH$_2$), 46.7 (CH$_2$), 42.9 (CH), 39.3 (CH$_2$), 28.4 (CH$_3$), 17.3 (CH$_3$), 12.7 (CH$_3$); *m/z* (EI) 566 (M$^+$), 568 (M$^+$+2); (CI) 567 (MH$^+$), 569 (MH$^+$+2); (Found MH$^+$, 567.2372. $C_{30}H_{35}{}^{35}ClN_4O_5$ requires MH$^+$, 567.2374); COSY spectra exhibited a good correlation with the proposed structure.

3-[4-(2-{[4-(2-Chloro-4-nitrophenylazo)-phenyl]-ethylamino}-ethoxy)-phenyl]-2-methylpropionic acid, **108**.[79]



To a solution of TIS:TFA, 1:49 (5 ml) was added a solution of **107** (354 mg, 0.63 mmol) in dichloromethane (1 ml). The reaction mixture was stirred at room temperature for 10 minutes after which time TLC (ethyl acetate:hexane, 2:3) indicated that the starting material had been consumed. The reaction mixture was concentrated under reduced pressure and the product isolated *via* flash column chromatography to give **108** as a red oil (258 mg, 0.51 mmol, 81%). ν_{max}(Nujol)/cm^{-1} 1707, 1601, 1515, 1339; δ_H(300 MHz; $CDCl_3$) 8.35 (1 H, d, *J* 2.5, ArC**H**), 8.11 (1 H, dd, *J* 8.9, 2.5, ArC**H**), 7.92 (2 H, d, J_{AB} 9.0, ArC**H**), 7.75 (1 H, d, *J* 8.9, ArC**H**), 7.08 (2 H, d, J_{AB} 9.0, ArC**H**), 6.79 (4 H, d, J_{AB} 9.0, ArC**H**), 4.16 (2 H, t, *J* 5.8, ArOC**H**$_2$CH$_2$), 3.85 (2 H, t, *J* 5.8, ArOCH$_2$C**H**$_2$), 3.61 (2 H, q, *J* 7.1, C**H**$_2$Me), 3.01-2.93 (1 H, m, ArCH$_2$C**H**), 2.74-2.58 (2 H, m, ArC**H**$_2$CH), 1.29 (3 H, t, *J* 7.1, CH$_2$**Me**), 1.15 (3 H, d, *J* 6.6, CH**Me**); δ_C(75 MHz; $CDCl_3$) 176.0 (C), 157.3 (C), 153.5 (C), 152.2 (C), 147.5 (C), 144.7 (C), 134.3 (C), 132.8 (C), 130.5 (2 CH), 127.4 (CH), 126.4 (CH), 123.0 (CH), 118.4 (CH), 115.7 (CH), 114.7 (2CH), 111.9 (2CH), 65.7 (CH_2), 50.3 (CH_2), 46.7 (CH_2), 41.8 (CH), 38.9 (CH_2), 16.9 (CH_3), 12.7 (CH_3); *m/z* (EI) 510 (M^+), 512 (M^++2); (CI) 511 (MH^+), 513 (MH^++2); (Found MH^+, 511.1751. $C_{26}H_{27}{}^{35}ClN_4O_5$ requires MH^+, 511.1748). COSY spectra exhibited a good correlation with the proposed structure.

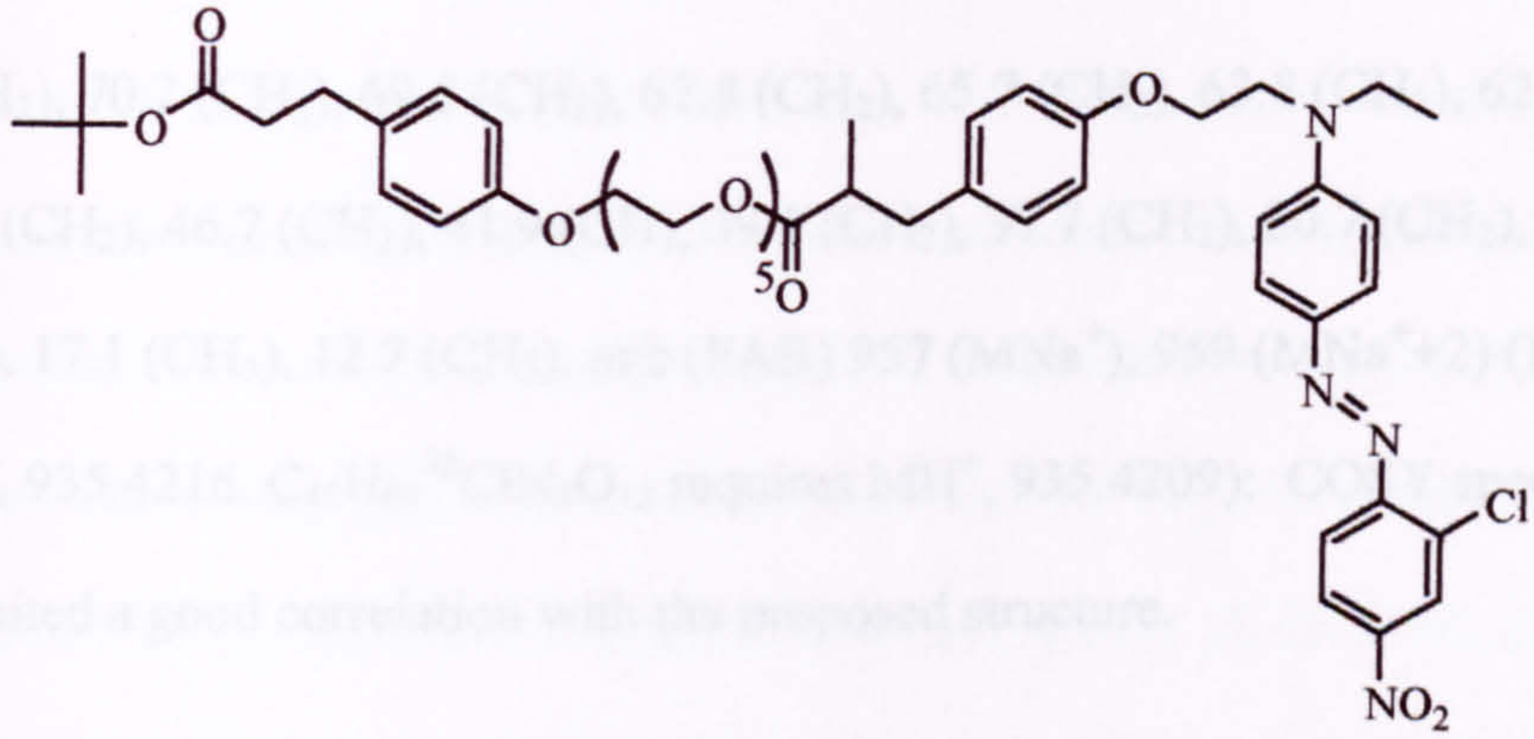
3-[4-(2-{[4-(2-Chloro-4-nitrophenylazo)-phenyl]-ethylamino}-ethoxy)-phenyl]-2-methylpropionic acid 2-(2-{2-[2-(2-hydroxy-ethoxy)-ethoxy]-ethoxy}-ethoxy)-ethyl ester, **109**.[55-57]



To a solution of **108** (200 mg, 0.39 mmol) in dichloromethane (10 ml) was added triphenylphosphine (113 mg, 0.43 mmol, 1.1 eq), diethyl azodicarboxylate (75 mg, 0.43 mmol, 1.1 eq) and pentaglycol (0.11 ml, 0.51 mmol, 1.3 eq). The reaction mixture was stirred at room temperature for 4 hours after which time TLC indicated that the starting material had been consumed. The reaction mixture was diluted with further dichloromethane (20 ml), washed with water, (3 x 20 ml), dried ($MgSO_4$) and concentrated under reduced pressure. The product was isolated *via* flash column chromatography to give **109** as a red oil (160 mg, 0.22 mmol, 56%). ν_{max}(Nujol)/cm^{-1}, 1731, 1600, 1514, 1337; δ_H(300 MHz; $CDCl_3$) 8.39 (1 H, d, *J* 2.4, ArC**H**), 8.15 (1 H, dd, *J* 8.9, 2.4, ArC**H**), 7.95 (2 H, d, J_{AB} 9.3, ArC**H**), 7.78 (1 H, d, *J* 8.9, ArC**H**), 7.08 (2 H, d, J_{AB} 8.7, ArC**H**), 6.81 (2 H, d, J_{AB} 9.3, ArC**H**), 6.79 (2 H, d, J_{AB} 8.7, ArC**H**), 4.24-4.15 (4 H, m, ArOC$\mathbf{H}_2$C$\mathbf{H}_2$), 3.86 (2 H, t, *J* 5.8, HOC$\mathbf{H}_2$CH_2), 3.73-3.58 (20 H, m, C$\mathbf{H}_2$), 2.99-2.93 (1 H, m, ArCH_2C**H**), 2.75-2.58 (2 H, m, ArC$\mathbf{H}_2$CH), 1.30 (3 H, t, *J* 7.1, CH_2**Me**), 1.13 (3 H, d, *J* 6.8, CH**Me**); δ_C(75 MHz; $CDCl_3$) 176.4 (C), 157.3 (C), 153.5 (C), 152.1 (C), 147.5 (C), 144.7 (C), 134.3 (C), 132.4 (C), 130.5 (2CH),

127.4 (CH), 126.4 (CH), 123.0 (CH), 118.4 (2CH), 114.6 (2CH), 111.9 (2CH), 72.9 (CH_2), 71.0 ($4CH_2$), 70.7 ($2CH_2$), 69.5 (CH_2), 65.7 (CH_2), 63.8 (CH_2), 62.1 (CH_2), 50.4 (CH_2), 46.7 (CH_2), 41.9 (CH), 39.1 (CH_2), 17.0 (CH_3), 12.7 (CH_3); *m/z* (FAB) 753 (MNa^+), 755 ($MNaH^+$+2) (Found: MH^+, 731.3053. $C_{36}H_{47}{}^{35}ClN_4O_{10}$ requires MH^+, 731.3059); COSY spectra exhibited a good correlation with the proposed structure.

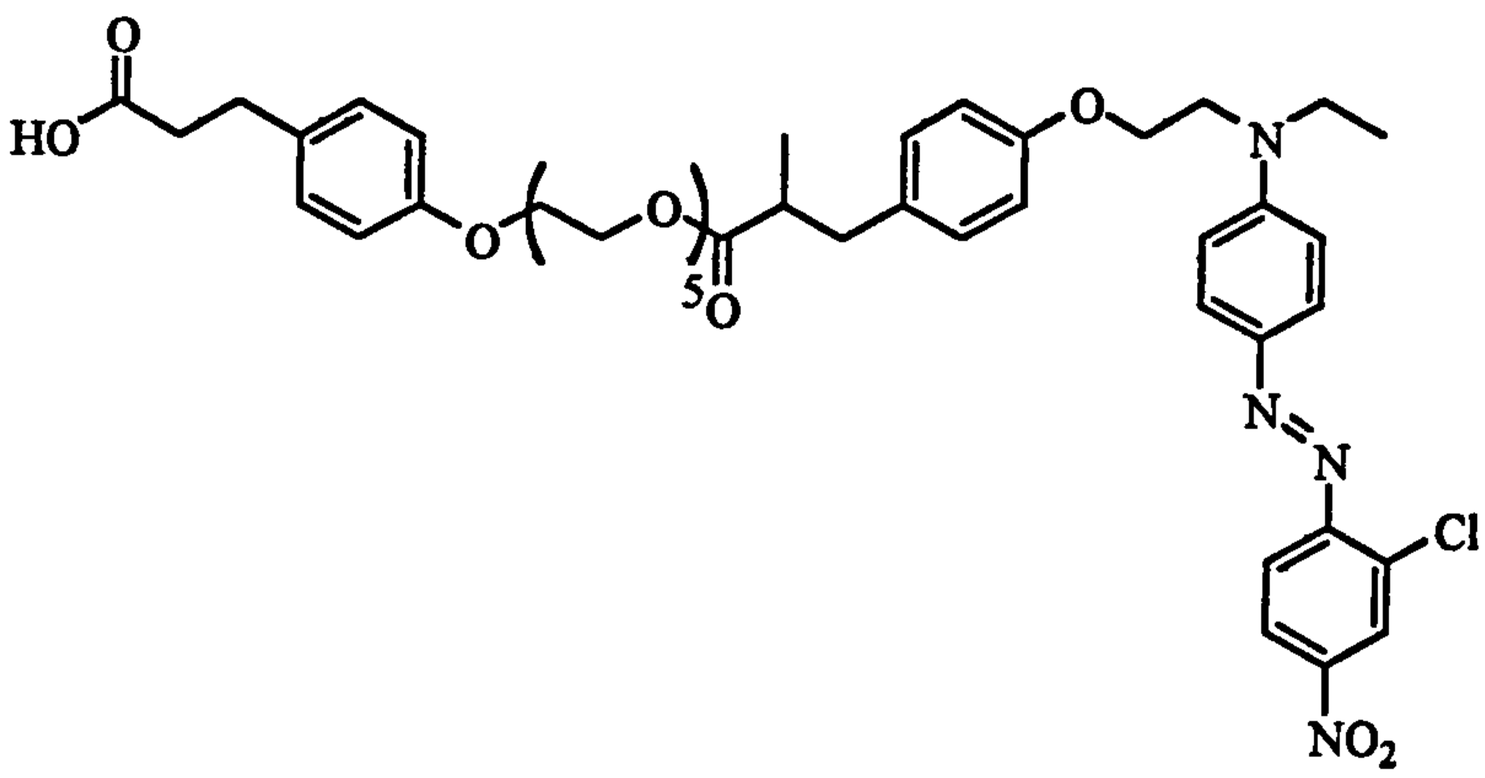
3-[4-(2-{[4-(2-Chloro-4-nitrophenylazo)-phenyl]-ethylamino}-ethoxy)-phenyl]-2-methylpropionic acid 2-{2-[2-(2-{2-[4-(2-tert-butoxycarbonylethyl)-phenoxy]-ethoxy}-ethoxy)-ethoxy]-ethoxy}-ethyl ester, **110**.[55-57]



To a solution of **109** (105 mg, 144 μmol) in dichloromethane was added triphenylphosphine (41.4 mg, 158 μmol, 1.1 eq), diethyl azodicarboxylate (28 mg, 158 μmol, 1.1 eq) and **62** (32 mg, 144 μmol). The reaction mixture was stirred at room temperature for 2 hours after which time TLC (ethyl acetate:hexane, 1:4) indicated that the starting material had been consumed. The reaction mixture was concentrated under reduced pressure and the product isolated *via* flash column chromatography (ethyl acetate:hexane, 1:4) to give **110**

(79 mg, 85 μmol, 59%); δ_H(300 MHz; $CDCl_3$) 8.38 (1 H, d, *J* 2.5, ArC**H**), 8.14 (1 H, dd, *J* 9.0, 2.5, ArC**H**), 7.95 (2 H, d, J_{AB} 9.2, ArC**H**), 7.78 (1 H, d, *J* 9.0, ArC**H**), 7.09 (2 H, d, J_{AB} 8.5, ArC**H**), 7.07 (2 H, d, J_{AB} 8.7, ArC**H**), 6.82 (2 H, d, J_{AB} 8.7, ArC**H**), 6.80 (2 H, d, J_{AB} 9.2, ArC**H**), 7.68 (2 H, d, J_{AB} 8.5, ArC**H**), 4.24-4.14 (4 H, m, ArOC$\mathbf{H}_2$C$\mathbf{H}_2$), 4.09 (2 H, t, *J* 4.9, HOC$\mathbf{H}_2$CH_2), 3.87-3.81 (4 H, m, C$\mathbf{H}_2$), 3.76-3.59 (16 H, m, C$\mathbf{H}_2$), 2.99-2.92 (1 H, m, ArCH_2C**H**), 2.83 (2 H, t, *J* 7.8, ArCH_2C$\mathbf{H}_2$), 2.75-2.57 (2 H, m, ArC$\mathbf{H}_2$CH), 2.49 (2 H, t, *J* 7.8, ArC$\mathbf{H}_2$CH_2), 1.41 (9 H, s, C$\mathbf{Me}_3$), 1.29 (3 H, t, *J* 7.0, CH_2**Me**), 1.13 (3 H, d, *J* 6.8, CH**Me**); δ_C(75 MHz; $CDCl_3$) 176.4 (C), 172.8 (C), 164.0 (C), 157.6 (C), 157.3 (C), 153.5 (C), 152.1 (C), 147.5 (C), 144.7 (C), 134.3 (C), 133.5 (C), 132.4 (C), 130.5 (2 CH), 129.6 (2 CH), 127.4 (CH), 126.4 (CH), 123.0 (CH), 118.4 (2 CH), 114.9 (2 CH), 114.6 (2 CH), 111.9 (2 CH), 80.7 (CH_2), 71.2 (CH_2), 71.0 (3CH_2), 70.2 (CH_2), 69.5 (CH_2), 67.8 (CH_2), 65.7 (CH_2), 63.8 (CH_2), 62.7 (CH_2), 50.4 (CH_2), 46.7 (CH_2), 41.9 (CH), 39.1 (CH_2), 37.7 (CH_2), 30.7 (CH_2), 28.5 (CH), 17.1 (CH_3), 12.7 (CH_3); *m/z* (FAB) 957 (MNa^+), 959 (MNa^++2) (Found: MH^+, 935.4216. $C_{49}H_{63}{}^{35}ClN_4O_{12}$ requires MH^+, 935.4209); COSY spectra exhibited a good correlation with the proposed structure.

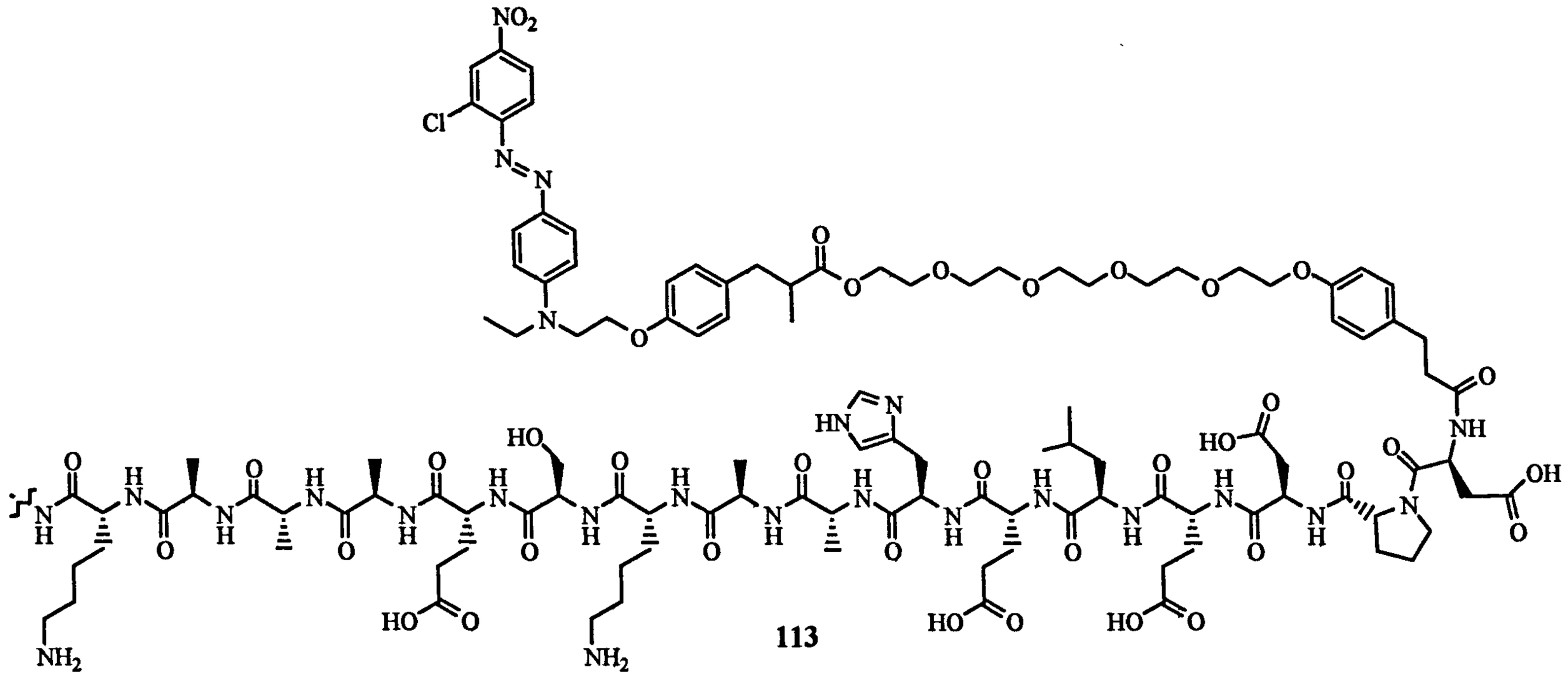
3-[4-(2-{[4-(2-Chloro-4-nitrophenylazo)-phenyl]-ethylamino}-ethoxy)-phenyl]-2-methylpropionic acid 2-{2-[2-(2-{2-[4-(2-carboxyethyl)-phenoxy]-ethoxy}-ethoxy)-ethoxy]-ethoxy}-ethyl ester, **111**.[79]



To a solution of TIS:TFA, 1:49 (3 ml) was added a solution of **110** (20 mg, 21 μmol) in dichloromethane (0.5 ml). The reaction mixture was stirred at room temperature for 10 minutes after which time TLC (ethyl acetate:hexane, 2:3) indicated that the starting material had been consumed. The reaction mixture was concentrated under reduced pressure and the product isolated *via* flash column chromatography to give **111** as a red oil (14.5 mg, 16.5 μmol, 79%); δ_H(300 MHz; $CDCl_3$) 8.38 (1 H, d, *J* 2.5, ArC**H**), 8.14 (1 H, dd, *J* 9.0, 2.5, ArC**H**), 7.95 (2 H, d, J_{AB} 9.2, ArC**H**), 7.78 (1 H, d, *J* 9.0, ArC**H**), 7.09 (2 H, d, J_{AB} 8.5, ArC**H**), 7.07 (2 H, d, J_{AB} 8.7, ArC**H**), 6.82 (2 H, d, J_{AB} 8.7, ArC**H**), 6.80 (2 H, d, J_{AB} 9.2, ArC**H**), 7.68 (2 H, d, J_{AB} 8.5, ArC**H**), 4.24-4.14 (4 H, m, ArOC$\mathbf{H_2}$C$\mathbf{H_2}$), 4.09 (2 H, t, *J* 4.9, HOC$\mathbf{H_2}$CH_2), 3.87-3.81 (4 H, m, C$\mathbf{H_2}$), 3.76-3.59 (16 H, m, C$\mathbf{H_2}$), 2.99-2.92 (1 H, m, ArCH_2C**H**), 2.83 (2 H, t, *J* 7.8, ArCH_2CH_2), 2.75-2.57 (2 H, m, ArC$\mathbf{H_2}$CH), 2.49 (2 H, t, *J* 7.8, ArC$\mathbf{H_2}$CH_2), 1.29 (3 H, t, *J* 7.0, CH_2**Me**), 1.11 (3 H, d, *J* 6.8, CH**Me**); δ_C(75 MHz; $CDCl_3$) 176.4 (C), 172.8 (C), 157.6 (C), 157.3 (C), 153.5 (C), 152.1 (C), 147.5 (C), 144.7

(C), 134.3 (C), 133.5 (C), 132.4 (C), 130.5 (2 CH), 129.6 (2 CH), 127.4 (CH), 126.4 (CH), 123.0 (CH), 118.4 (2 CH), 114.9 (2 CH), 114.6 (2 CH), 111.9 (2 CH), 80.7 (CH_2), 71.2 (CH_2), 71.0 ($3CH_2$), 70.2 (CH_2), 69.5 (CH_2), 67.8 (CH_2), 65.7 (CH_2), 63.8 (CH_2), 62.7 (CH_2), 50.4 (CH_2), 46.7 (CH_2), 41.9 (CH), 39.1 (CH_2), 37.7 (CH_2), 30.7 (CH_2), 17.1 (CH_3), 12.7 (CH_3); *m/z* (FAB) 901 (MNa^+), 903 (MNa^++2) (Found: MH^+, 879.8577. $C_{45}H_{55}{}^{35}ClN_4O_{12}$ requires MH^+, 879.3583); COSY spectra exhibited a good correlation with the proposed structure.

Synthesis of the protected and deprotected peptidyl chiral dye-linker systems, **112** *and* **113**.



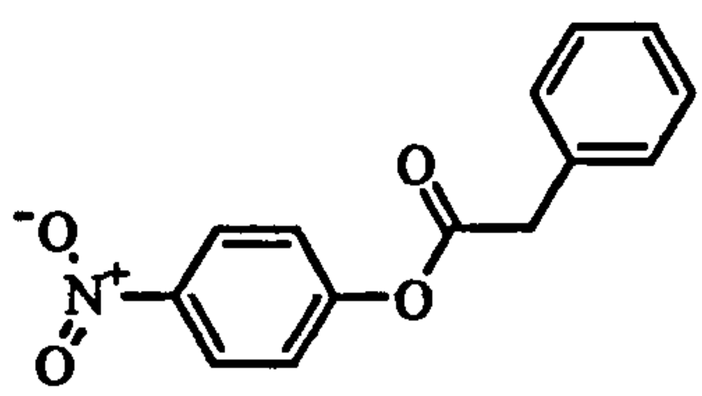
113

Preparation for the attaching the chiral dye-linker to the terminal amino group of the peptide, followed by deprotection and partial cleavage of the system from the polymer supported was:

a) To a suspension of the peptide on Tentagel **40** (10 mg) in dimethylformamide (1 ml) was added the dye-linker **111** (10 mg, 11.4 μmol, excess), PyBOP (30 mg, 57.7 μmol, excess), HOBt (30 mg, 222 μmol, excess) and DIPEA (3 drops, ~30 mg, 233 μmol, excess), and gently mixed by bubbling nitrogen through at room temperature for 3 hours. The resin was then isolated by filtration and washed with dimethylformamide (2 x 10 ml), dichloromethane (2 x 10 ml), methanol (2 x 10 ml) and dried under reduced pressure to give a black resin **112** (11 mg).

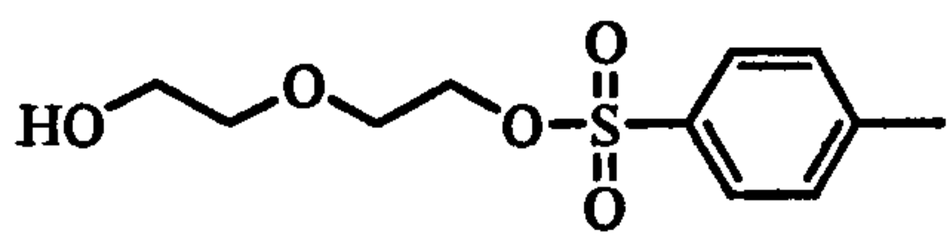
b) The peptidyl dye-linker system **112** (9 mg) was suspended in triisopropylamine:TFA, 1:49 (3 ml) and gently mixed by bubbling nitrogen through at room temperature for 2 hours. The resin was isolated by filtration, washed with methanol (2 x 10 ml), dichloromethane (2 x 10 ml) and dried under reduced pressure to give the supported unprotected peptide chiral dye-linker system **113** as a black resin (7 mg). The filtrate was dried under reduced pressure to give a red powder (1 mg), the result of cleavage of the 10% Rink resin.

Phenylacetic acid 4-nitrophenyl ester, **103**.[55-57]



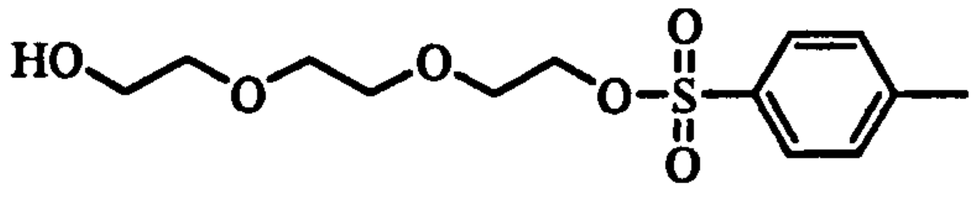
To a solution of phenyl acetic acid (300 mg, 2.21 mmol), triphenylphosphine (578 mg, 2.21 mmol) and diethyl azodicarboxylate (0.35 ml, 2.21 mmol) in toluene (20 ml) was added *p*-nitrophenol (307 mg, 2.21 mmol). The reaction mixture was stirred for 12 hours at room temperature after which TLC (ethyl acetate:hexane, 1:4) indicated that the phenyl acetic acid had been consumed. The toluene was removed at 50°C under reduced pressure to give a pale yellow oil which was redissolved in ether, washed with $NaHCO_3$ (3 x 30 ml), HCl (2 M 3 x 30 ml), water (3 x 30 ml), dried ($MgSO_4$) and concentrated in *vacuo*. The product was eluted by flash column chromatography (ethyl acetate:hexane, 1:9) to give **103** as a clear very pale yellow oil, (352 mg, 1.37 mmol, 62%). ν_{max}(Nujol)/cm^{-1}, 1764, 1614, 1524, 1347; δ_H(300 MHz; $CDCl_3$) 8.21 (2 H, d, J_{AB} 9.0, ArC**H**), 7.38-7.28 (5 H, m, ArC**H**), 7.24 (2 H, d, J_{AB} 9.0, ArC**H**), 3.89 (2 H, s, ArC$\mathbf{H}_2$); δ_C(75 MHz; $CDCl_3$) 169.5 (C), 155.8 (C), 145.7 (C), 133.1 (C), 129.7 (2 CH), 129.3 (2 CH), 128.1 (CH), 125.6 (2 CH), 122.8 (2 CH), 41.7 (CH_2); *m/z* (EI) 257 (M^+); (CI) 275 (MNH_4^+) (Found: MH^+, 258.0766, MNH_4^+, 275.1033. $C_{14}H_{11}NO_4$ requires MH^+, 258.0766, MNH_4^+, 275.1032);

Toluene-4-sulfonic acid 2-(2-hydroxyethoxy)-ethyl ester, **119**.[45]



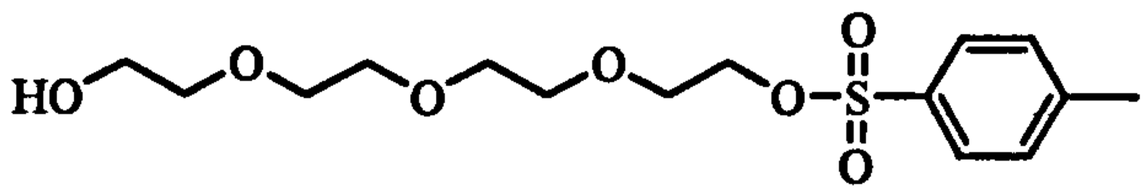
To a solution of diethylene glycol (15 ml, 158.0 mmol, 5 eq) in dichloromethane (150 ml) triethylamine (13.1 ml, 94.8 mmol, 3 eq) and a catalytic amount of dimethylaminopyridine (1-2 crystals), *p*-toluenesulfonyl chloride (6.03 g, 31.6 mmol, 1 eq) was carefully added at 0°C. The reaction mixture was allowed to warm to room temperature and stirred for 8 hours after which TLC (ethyl acetate:hexane, 4:1) indicated that the *p*-toluenesulfonyl chloride had been consumed. The dichloromethane was removed under *vacuo* to give a pale yellow solid which was redissolved in diethyl ether. This was washed with water (3 x 30 ml) dried ($MgSO_4$) and concentrated in *vacuo*. The product was eluted by flash column chromatography (ethyl acetate:hexane, 2:3) to give **119** as a clear colourless oil, (3.56 g, 13.7 mmol, 43%). ν_{max}(Nujol)/cm^{-1} 3401, 1597; δ_H(300 MHz; $CDCl_3$) 7.80 (2 H, d, J_{AB} 8.3, ArC**H**), 7.36 (2 H, d, J_{AB} 8.3, ArC**H**), 4.19 (2 H, t, *J* 4.6, CH_2CH_2OTs), 3.69 (2 H, t, *J* 4.6, CH_2CH_2OTs), 3.67 (2 H, t, *J* 3.7, $HOCH_2CH_2$), 3.53 (2 H, t, *J* 3.7, $HOCH_2CH_2$), 2.45 (3 H, s, Ar**Me**); δ_C(75 MHz; $CDCl_3$) 145.4 (C), 133.3 (C), 130.3 (2 CH), 128.3 (2 CH), 72.9 (CH_2), 69.6 (CH_2), 68.9 (CH_2), 62.0 (CH_2), 22.0 (CH_3); *m/z* (CI) 261 (MH^+), 278 (MNH_4^+) (Found: MH^+, 261.0797. $C_{11}H_{16}O_5S$ requires MH^+, 261.0795); COSY spectra exhibited a good correlation with the proposed structure.

Toluene-4-sulfonic acid 2-[2-(2-hydroxyethoxy)-ethoxy]-ethyl ester, **120**.[45]



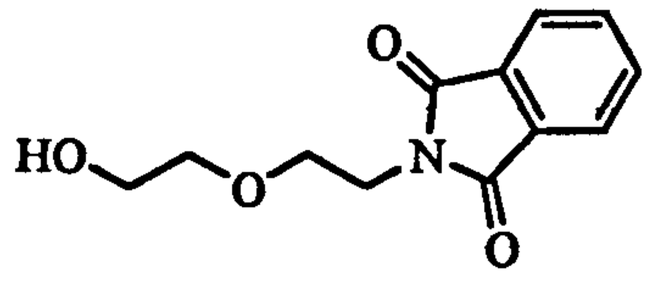
To a solution of triethylene glycol (21.1 ml, 158.0 mmol, 5 eq) in dichloromethane (150 ml), triethylamine (13.1 ml, 94.8 mmol, 3 eq) and a catalytic amount of dimethylaminopyridine (1-2 crystals), *p*-toluenesulfonyl chloride (6.03 g, 31.6 mmol) was carefully added at 0°C. The reaction mixture was allowed to warm to room temperature and stirred for 8 hours after which TLC (ethyl acetate:hexane, 4:1) indicated that the *p*-toluenesulfonyl chloride had been consumed. The dichloromethane was removed under *vacuo* to give a pale yellow solid which was redissolved in diethyl ether. This was washed with water (3 x 30 ml) dried ($MgSO_4$) and concentrated in *vacuo*. The product was eluted by flash column chromatography (ethyl acetate:hexane, 3:2) to give **120** as a clear colourless oil, (3.70 g, 12.2 mmol, 39%). ν_{max}(Nujol)/cm^{-1} 3417, 1597; δ_H(300 MHz; $CDCl_3$) 7.80 (2 H, d, J_{AB} 8.4, ArC**H**), 7.35 (2 H, d, J_{AB} 8.4, ArC**H**), 4.17 (2 H, t, *J* 4.8, CH_2CH_2OTs), 3.73-3.69 (4 H, m, CH_2), 3.61-3.56 (6 H, m, CH_2), 2.67 (1 H, s, $HOCH_2CH_2$), 2.45 (3 H, s, Ar**Me**); δ_C(75 MHz; $CDCl_3$) 145.3 (C), 133.3 (C), 130.2 (2 CH), 128.3 (2 CH), 72.9 (CH_2), 71.1 (CH_2), 70.6 (CH_2), 69.6 (CH_2), 69.1 (CH_2), 62.1 (CH_2), 22.0 (CH_3); *m/z* (CI) 305 (MH^+), 322 (MNH_4^+) (Found: MNH_4^+, 322.1321. $C_{13}H_{20}O_6S$ requires MNH_4^+, 322.1324); COSY spectra exhibited a good correlation with the proposed structure.

Toluene-4-sulfonic acid 2-{2-[2-(2-hydroxyethoxy)-ethoxy]-ethoxy}-ethyl ester, **121**.[45]



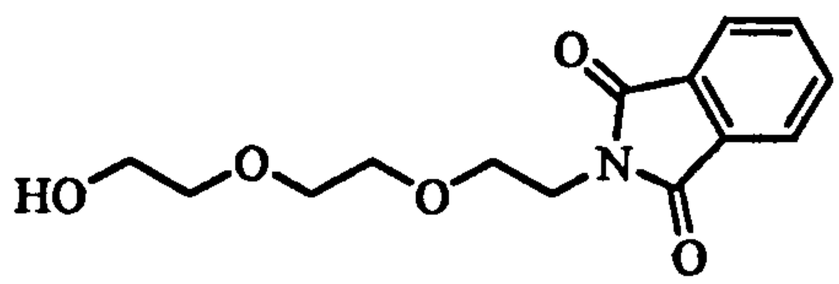
To a solution of tetraethylene glycol (27.3 ml, 158.0 mmol, 5 eq) in dichloromethane (150 ml), triethylamine (13.1 ml, 94.8 mmol, 3 eq) and a catalytic amount of dimethylaminopyridine (1-2 crystals), *p*-toluenesulfonyl chloride (6.03 g, 31.6 mmol) was carefully added at 0°C. The reaction mixture was allowed to warm to room temperature and stirred for 8 hours after which TLC (ethyl acetate:hexane, 4:1) indicated that the *p*-toluenesulfonyl chloride had been consumed. The dichloromethane was removed under *vacuo* to give a pale yellow solid which was redissolved in diethyl ether. This was washed with water (3 x 30 ml) dried ($MgSO_4$) and concentrated in *vacuo*. The product was eluted by flash column chromatography (ethyl acetate:hexane, 4:1) to give **121** as a clear colourless oil, (4.38 g, 12.6 mmol, 40%). ν_{max}(Nujol)/cm^{-1} 3426, 1556; δ_H(300 MHz; $CDCl_3$) 7.80 (2 H, d, J_{AB} 8.4, ArC**H**), 7.35 (2 H, d, J_{AB} 8.4, ArC**H**), 4.16 (2 H, t, *J* 4.7, CH_2CH_2OTs), 3.72-3.57 (14 H, m, CH_2), 3.37 (1 H, s, **H**OCH_2CH_2), 2.45 (3 H, s, Ar**Me**); δ_C(75 MHz; $CDCl_3$) 144.6 (C), 132.6 (C), 129.6 (2 CH), 127.2 (2 CH), 72.2 (CH_2), 70.4 (CH_2), 70.3 (CH_2), 69.9 (CH_2), 69.6 (CH_2), 69.1 (CH_2), 68.4 (CH_2), 61.3 (CH_2), 21.2 (CH_3); *m/z* (EI) 348 (M^+), 349(MH^+); (CI) 366 (MNH_4^+); (Found MNH_4^+, 366.1591. $C_{15}H_{24}O_7S$ requires MNH_4^+, 366.1586); COSY spectra exhibited a good correlation with the proposed structure.

2-[2-(2-Hydroxyethoxy)-ethyl]-isoindole-1,3-dione, **122**.[100]



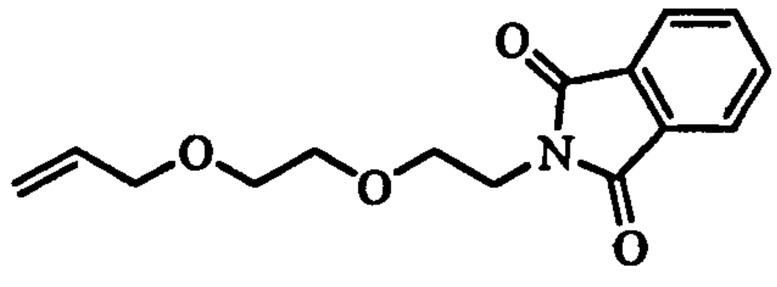
To a solution of diethylene glycol tosylate **119** (3.5 g, 13.5 mmol) in dimethylformamide (50 ml) potassium phthalimide (2.74 g, 14.8 mmol, 1.1 eq) was added. The reaction mixture was stirred at 60°C for 12 hours after which time TLC (80% ethyl acetate in hexane, and PMA dip to visualise) indicated that the starting material had been consumed. The reaction mixture was allowed to cool down to room temperature and 40 ml of water was added. The product was extracted with ether (5 x 20 ml) and the organic fraction combined and washed with water (20 ml). This was then dried ($MgSO_4$) and concentrated in *vacuo* to give a clear pale yellow oil. The product was isolated by flash column chromatography (ethyl acetate:hexane, 3:2) to give **122** as a clear colourless oil, (2.54 g, 10.8 mmol, 80%). ν_{max}(Nujol)/cm^{-1} 3467, 1774, 1772, 1614; δ_H(300 MHz; $CDCl_3$) 7.89-7.82 (2 H, m, ArC**H**), 7.76-7.70 (2 H, m, ArC**H**), 3.92 (2 H, t, *J* 5.5, PhthNC**H**$_2$CH$_2$), 3.75 (2 H, t, *J* 5.5, PhNCH$_2$C**H**$_2$), 3.69 (2 H, t, *J* 4.8, CH$_2$C**H**$_2$OH), 3.61 (2 H, t, *J* 4.8, C**H**$_2$CH$_2$OH), 2.42 (1 H, brs, CH$_2$CH$_2$O**H**); δ_C(75 MHz; $CDCl_3$) 168.9 (2 C*i*), 134.5 (2 CH), 132.4 (2 C*i*), 123.7 (2 CH), 72.6 (CH_2), 68.8 (CH_2), 62.1 (CH_2), 38.0 (CH_2); *m/z* (CI) 236 (MH^+), 253 (MNH_4^+) (Found: MH^+, 236.0920. $C_{12}H_{13}NO_4$ requires MH^+, 236.0923); COSY spectra exhibited a good correlation with the proposed structure.

2-{2-[2-(2-Hydroxyethoxy)-ethoxy]-ethyl}-isoindole-1,3-dione, **123**.[100]



To a solution of triethylene glycol tosylate **120** (3.5 g, 11.5 mmol) in dimethylformamide (50 ml) was added potassium phthalimide (2.34 g, 12.7 mmol, 1.1 eq). The reaction mixture was stirred at 60°C for 12 hours after which time TLC (ethyl acetate:hexane, 4:1, and PMA dip to visualise) indicated that the starting material had been consumed. The reaction mixture was allowed to cool down to room temperature and 40 ml of water was added. The product was extracted with ether (5 x 20 ml) and the organic fraction combined and washed with water (20 ml). This was then dried ($MgSO_4$) and concentrated in *vacuo* to give a clear pale yellow oil. The product was isolated by flash column chromatography (ethyl acetate:hexane, 4:1) to give **123** as a clear colourless oil, (2.44 g, 8.75 mmol, 76%). ν_{max}(Nujol)/cm^{-1} 3465, 1770, 1711, 1614; δ_H(300 MHz; $CDCl_3$) 7.80-7.74 (2 H, m, ArC**H**), 7.68-7.61 (2 H, m, ArC**H**), 3.83 (2 H, t, *J* 5.6, PhthNC**H**$_2$CH$_2$), 3.68 (2 H, t, *J* 5.6, PhthNCH$_2$C**H**$_2$), 3.60-3.51 (6 H, m, C**H**$_2$), 3.46 (2 H, t, *J* 4.3, C**H**$_2$CH$_2$OH), 2.74 (1 H, t, *J* 6.0, CH$_2$CH$_2$O**H**); δ_C(75 MHz; $CDCl_3$) 168.7 (2 C*i*), 134.9 (2 CH), 134.4 (2 C*i*), 123.7 (2 CH), 72.9 (CH_2), 70.7 (CH_2), 70.3 (CH_2), 68.3 (CH_2), 62.1 (CH_2), 37.6 (CH_2); *m/z* (CI) 280 (MH^+), 297 (MNH_4^+) (Found: MH^+, 280.1188. $C_{14}H_{17}NO_5$ requires MH^+, 280.1185); COSY spectra exhibited a good correlation with the proposed structure.

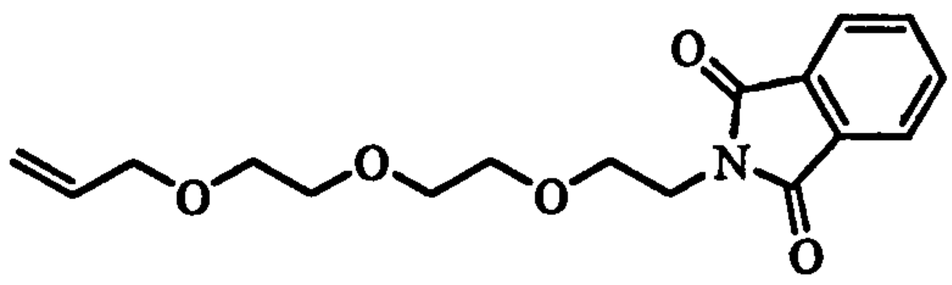
2-[2-(2-Allyloxyethoxy)-ethyl]-isoindole-1,3-dione, **125**.



To a solution of diethylene glycol phthalimide **122** (2.0 g, 8.51 mmol) in tetrahydrofuran (25 ml), cesium carbonate (catalytic) and sodium hydride [50% w/w dispersion in mineral oil], (449 mg, 9.36 mmol, 1.1 eq) was carefully added at 0°C. Allyl bromide (1.08 ml, 12.8 mmol, 1.5 eq) was then quickly added at 0°C, the reaction mixture allowed to warm to room temperature and stirred for 4 hours after which time TLC (ethyl acetate:hexane, 4:1, and PMA dip to visualise) indicated that the starting material had been consumed. This was then carefully quenched with water (2 ml), diluted with diethyl ether (30 ml), washed with water (3 x 30 ml), dried ($MgSO_4$) and concentrated in *vacuo*. The product was isolated by flash column chromatography (ethyl acetate:hexane, 1:4) to give **125** as a clear colourless oil, (1.44 g ,5.20 mmol, 61%). ν_{max}(Nujol)/cm^{-1} 1774, 1713; δ_H(300 MHz; $CDCl_3$) 7.88-7.82 (2 H, m, ArC**H**), 7.75-7.27 (2 H, m, ArC**H**), 5.84 (1 H, ddt, *J* 17.1, 10.4, 5.7, $CH_2C\mathbf{H}CH_2O$), 5.22 (1 H, ddt, *J* 17.1, 1.7, 1.7, *trans*-$C\mathbf{H}_2CHCH_2O$), 5.13 (1 H, ddt, *J* 10.4, 1.7, 1.3, *cis*-$C\mathbf{H}_2CHCH_2O$), 3.96 (2 H, dt, *J* 5.7, 1.3, $CH_2CHC\mathbf{H}_2O$), 3.91 (2 H, t, *J* 5.9, PhthNC$\mathbf{H}_2CH_2$), 3.76 (2 H, t, *J* 5.9, PhthN$CH_2C\mathbf{H}_2$), 3.68-3.62 (2 H, m, $OCH_2C\mathbf{H}_2$Oallyl), 3.59-3.54 (2 H, m, $OC\mathbf{H}_2CH_2$Oallyl); δ_C(75 MHz; $CDCl_3$) 168.7 (2 C*i*), 135.1 (CH), 134.3 (2 CH), 132.5 (2 C*i*), 123.6 (2 CH), 117.4, (CH_2), 72.6 (CH_2), 70.5 (CH_2), 69.8 (CH_2), 68.3 (CH_2), 37.6 (CH_2); *m/z* (CI) 276 (MH^+), 293 (MNH_4^+) (Found: MH^+,

276.1240. $C_{15}H_{17}NO_4$ requires MH^+, 276.1236); COSY spectra exhibited a good correlation with the proposed structure.

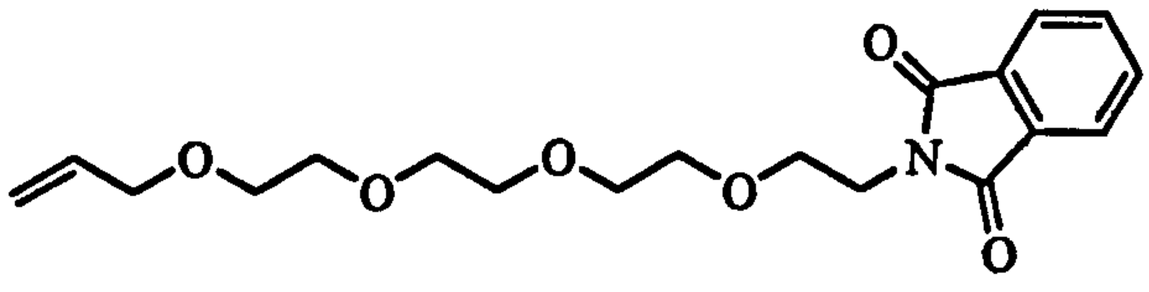
2-{2-[2-(2-Allyloxyethoxy)-ethoxy]-ethyl}-isoindole-1,3-dione, **126**.



To a solution of triethylene glycol phthalimide **123** (2.0 g, 7.17 mmol) in tetrahydrofuran (40 ml), cesium carbonate (catalytic) and sodium hydride [50% w/w dispersion in mineral oil], (378 mg, 7.89 mmol, 1.1 eq) was carefully added at 0°C. Allyl bromide (0.91 ml, 10.8 mmol, 1.5 eq) was then quickly added at 0°C, the reaction mixture allowed to warm to room temperature and stirred for 4 hours after which time TLC (ethyl acetate:hexane, 4:1, and PMA dip to visualise) indicated that the starting material had been consumed. This was then carefully quenched with water (2 ml), diluted with diethyl ether (30 ml), washed with water (3 x 30 ml), dried ($MgSO_4$) and concentrated in *vacuo*. The product was isolated by flash column chromatography (ethyl acetate:hexane, 2:3) to give **126** as a clear colourless oil, (1.34 g, 4.17 mmol, 58%). ν_{max}(Nujol)/cm^{-1} 2869, 1774, 1713; δ_H(300 MHz; $CDCl_3$) 7.88-7.81 (2 H, m, ArC**H**), 7.75-7.69 (2 H, m, ArC**H**), 5.89 (1 H, ddt, *J* 17.3, 10.4, 5.7, $CH_2C\mathbf{H}CH_2O$), 5.26 (1 H, ddt, *J* 17.1, 1.7, 1.7, *trans*-$C\mathbf{H}_2CHCH_2O$), 5.17 (1 H, ddt, *J* 10.4, 1.7, 1.3, *cis*-$C\mathbf{H}_2CHCH_2O$), 3.99 (2 H, dt, *J* 5.7, 1.3, $CH_2CHC\mathbf{H}_2O$), 3.90 (2 H, t, *J* 5.9, PhthNC$\mathbf{H}_2CH_2$), 3.75

(2 H, t, *J* 5.9, PhthNCH$_2$C**H**$_2$), 3.68-3.64 (2 H, m, OCH$_2$C**H**$_2$Oallyl), 3.63-3.59 (4 H, m, C**H**$_2$), 3.55-3.51 (2 H, m, OC**H**$_2$CH$_2$Oallyl); δ_C(75 MHz; $CDCl_3$) 168.6 (2 C*i*), 135.2 (CH), 134.3 (2 CH), 132.5 (2 C*i*), 123.6 (2 CH), 117.5 (CH_2), 72.6 (CH_2), 71.0 (CH_2), 71.0 (CH_2), 70.5 (CH_2), 69.8 (CH_2), 68.3 (CH_2), 37.6 (CH_2); *m/z* (CI) 320 (MH^+), 337 (MNH_4^+) (Found: MH^+, 320.1497. $C_{17}H_{21}NO_5$ requires MH^+, 320.1497); COSY spectra exhibited a good correlation with the proposed structure.

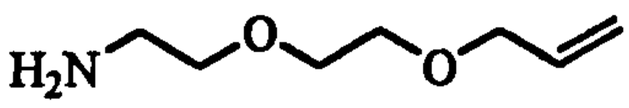
2-(2-{2-[2-(2-Allyloxyethoxy)-ethoxy]-ethoxy}-ethyl)-isoindole-1,3-dione, **127**.



To a solution of tetraethylene glycol tosylate **124** (3.5 g, 10.1 mmol) in dimethylformamide (40 ml) was added potassium phthalimide (2.05 g, 11.1 mmol, 1.1 eq). The reaction mixture was stirred at 60°C for 12 hours after which time TLC (ethyl acetate:hexane, 4:1, and PMA dip to visualise) indicated that the starting material had been consumed. The reaction mixture was allowed to cool to room temperature and further then cooled to 0°C. Allyl bromide (1.28 ml, 15.1 mmol, 1.5 eq), cesium carbonate (catalytic) and sodium hydride [50% w/w dispersion in mineral oil], (630 mg, 13.1 mmol, 1.3 eq) were carefully added and the reaction mixture was stirred at room temperature overnight after which TLC (ethyl acetate:hexane, 3:2, and PMA dip to visualise) indicated that the

intermediate product had been consumed. This was then carefully quenched with water (40 ml) and the product extracted with ether (5 x 20 ml). The organic fractions were combined, washed with water (20 ml), dried ($MgSO_4$) and concentrated in *vacuo* to give a clear pale yellow oil. The product was isolated by flash column chromatography (ethyl acetate:hexane, 2:3) to give **127** as a clear colourless oil, (1.56 g, 4.27 mmol, 42%). ν_{max}(Nujol)/cm^{-1}, 1774, 1713, 1394; δ_H(300 MHz; $CDCl_3$) 7.87-7.81 (2 H, m, ArC**H**), 7.75-7.69 (2 H, m, ArC**H**), 5.92 (1 H, ddt, *J* 17.3, 10.4, 5.7, $CH_2C\mathbf{H}CH_2O$), 5.27 (1 H, ddt, *J* 17.1, 1.7, 1.7, *trans*-$C\mathbf{H}_2CHCH_2O$), 5.17 (1 H, ddt, *J* 10.4, 1.7, 1.3, *cis*-$C\mathbf{H}_2CHCH_2O$), 4.02 (2 H, dt, *J* 5.7, 1.3, $CH_2CHC\mathbf{H}_2O$), 3.90 (2 H, t, *J* 5.8, $PhthNC\mathbf{H}_2CH_2$), 3.75 (2 H, t, *J* 5.8, $PhthNCH_2C\mathbf{H}_2$), 3.68-3.56 (12 H, m, $C\mathbf{H}_2$); δ_C(75 MHz; $CDCl_3$) 168.6 (2 C*i*), 135.1 (CH), 134.3 (2 CH), 132.5 (2 C*i*), 123.6 (2 CH), 117.4 (CH_2), 72.5 (CH_2), 70.9 (4 CH_2), 70.4 (CH_2), 69.7 (CH_2), 68.2 (CH_2), 37.6 (CH_2); *m/z* (EI) 364 (MH^+); (CI) 381 (MNH_4^+); (Found MNH_4^+, 381.2027. $C_{19}H_{25}NO_6$ requires MNH_4^+, 381.2026). COSY spectra exhibited a good correlation with the proposed structure.

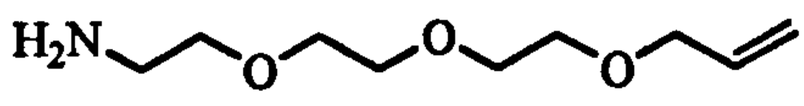
2-(2-Allyloxyethoxy)-ethylamine, **128**.[100]



To a solution of allyl diglycolphthalimide **125** (1.0 g, 3.64 mmol) in ethanol (50 ml) hydrazine monohydrate was added dropwise (approximately 200 mg, 4.0 mmol, excess) over a period of 30 minutes at which point TLC (ethyl

acetate:hexane, 2:3 and permanganate dip to visualise) indicated that the starting material had been consumed. Concentrated hydrochloric acid (10 drops) was carefully added, the reaction mixture was refluxed for 2 hours and then cooled to room temperature after which time TLC (methanol:dichloromethane, 1:9 and ninhydrin dip to visualise) indicated the presence of the product. The reaction mixture was then concentrated in *vacuo*, diluted with ethyl acetate (10 ml) and the product extracted with dilute hydrochloric acid (1 M, 2 x 5 ml). The aqueous extract was made basic with sodium hydroxide (4 M, 2 x 5 ml) and the product extracted with diethyl ether (4 x 10 ml). The organic fractions were combined and reduced under *vacuo* to give **128** as a clear pale yellow oil (380 mg, 2.62 mmol, 72%). ν_{max}(Nujol)/cm^{-1} 3408, 1652; δ_H(300 MHz; $CDCl_3$) 5.93 (1 H, ddt, *J* 17.2, 10.3, 5.7, CH_2CHCH_2O), 5.29 (1 H, ddt, *J* 17.2, 1.6, 1.6, *trans*-CH_2CHCH_2O), 5.19 (1 H, ddt, *J* 10.3, 1.6, 1.6, *cis*-CH_2CHCH_2O), 4.04 (2 H, dt, *J* 5.7, 1.6, CH_2CHCH_2O), 3.67-3.57 (6 H, m, CH_2), 3.52 (2 H, t, *J* 5.3, CH_2), 2.90 (2 H, br s, NH_2); δ_C(75 MHz; $CDCl_3$) 135.1 (2 CH), 117.6 (CH_2), 72.7 (CH_2), 70.7 (CH_2), 70.6 (CH_2), 69.8 (CH_2); *m/z* (CI) 146 (MH^+); COSY spectra exhibited a good correlation with the proposed structure.

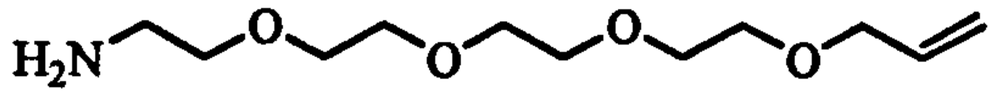
2-[2-(2-Allyloxyethoxy)-ethoxy]-ethylamine, **129**.[100]



To a solution of allyl triglycolphthalimide **126** (1.0 g, 3.13 mmol) in ethanol (50 ml) hydrazine monohydrate was added dropwise (approximately 200 mg, 4.0

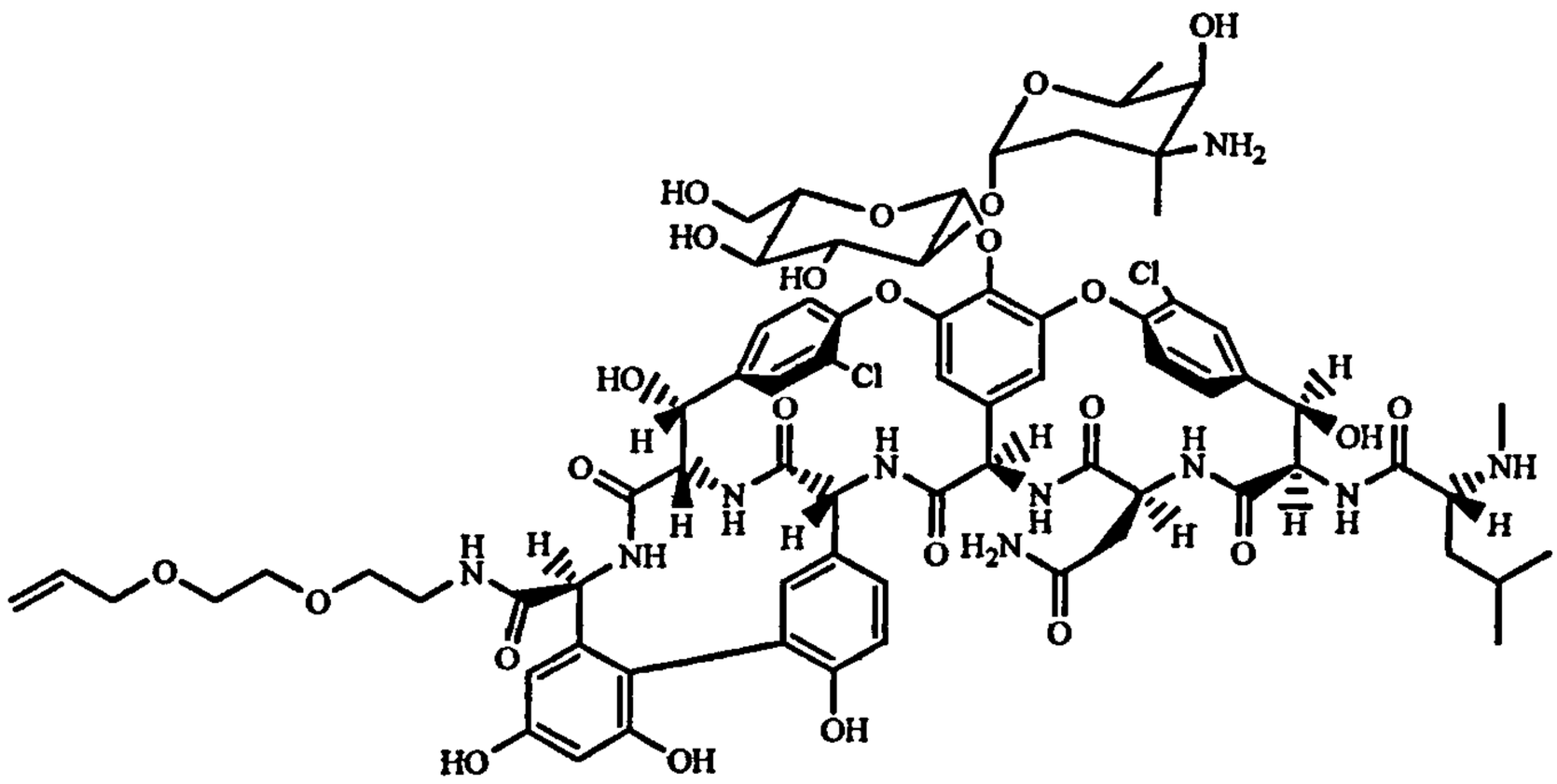
mmol, excess) over a period of 30 minutes at which point TLC (ethyl acetate:hexane, 2:3 and permanganate dip to visualise) indicated that the starting material had been consumed. Concentrated hydrochloric acid (10 drops) was carefully added, the reaction mixture was refluxed for 2 hours and then cooled to room temperature after which time TLC (methanol:dichloromethane, 1:9 and ninhydrin dip to visualise) indicated the presence of the product. The reaction mixture was then concentrated in *vacuo*, diluted with ethyl acetate (10 ml) and the product extracted with dilute hydrochloric acid (1 M, 2 x 5 ml). The aqueous extract was made basic with sodium hydroxide (4 M, 2 x 5 ml) and the product extracted with diethyl ether (4 x 10 ml). The organic fractions were combined and reduced under *vacuo* to give **129** as a clear pale yellow oil (444 mg, 2.35 mmol, 75%). ν_{max}(Nujol)/cm^{-1} 3415, 1652; δ_H(300 MHz; $CDCl_3$) 5.94 (1 H, ddt, *J* 17.3, 10.4, 5.8, CH_2**C**HCH_2O), 5.28 (1 H, ddt, *J* 17.3, 1.3, 1.3, *trans*-CH_2CHCH_2O), 5.18 (1 H, ddt, *J* 10.4, 1.3, 1.3, *cis*-CH_2CHCH_2O), 4.04 (2 H, dt, *J* 5.8, 1.3, CH_2CHCH_2O), 3.71-3.60 (12 H, m, CH_2), 3.35 (2 H, br s, NH_2); δ_C(75 MHz; $CDCl_3$) 135.1 (2 CH), 117.5 (CH_2), 72.6 (CH_2), 71.0 (3 CH_2), 70.7 (CH_2), 69.8 (CH_2); *m/z* (CI) 190 (MH^+); COSY spectra exhibited a good correlation with the proposed structure.

2-{2-[2-(2-Allyloxyethoxy)-ethoxy]-ethoxy}-ethylamine, **130**.[100]

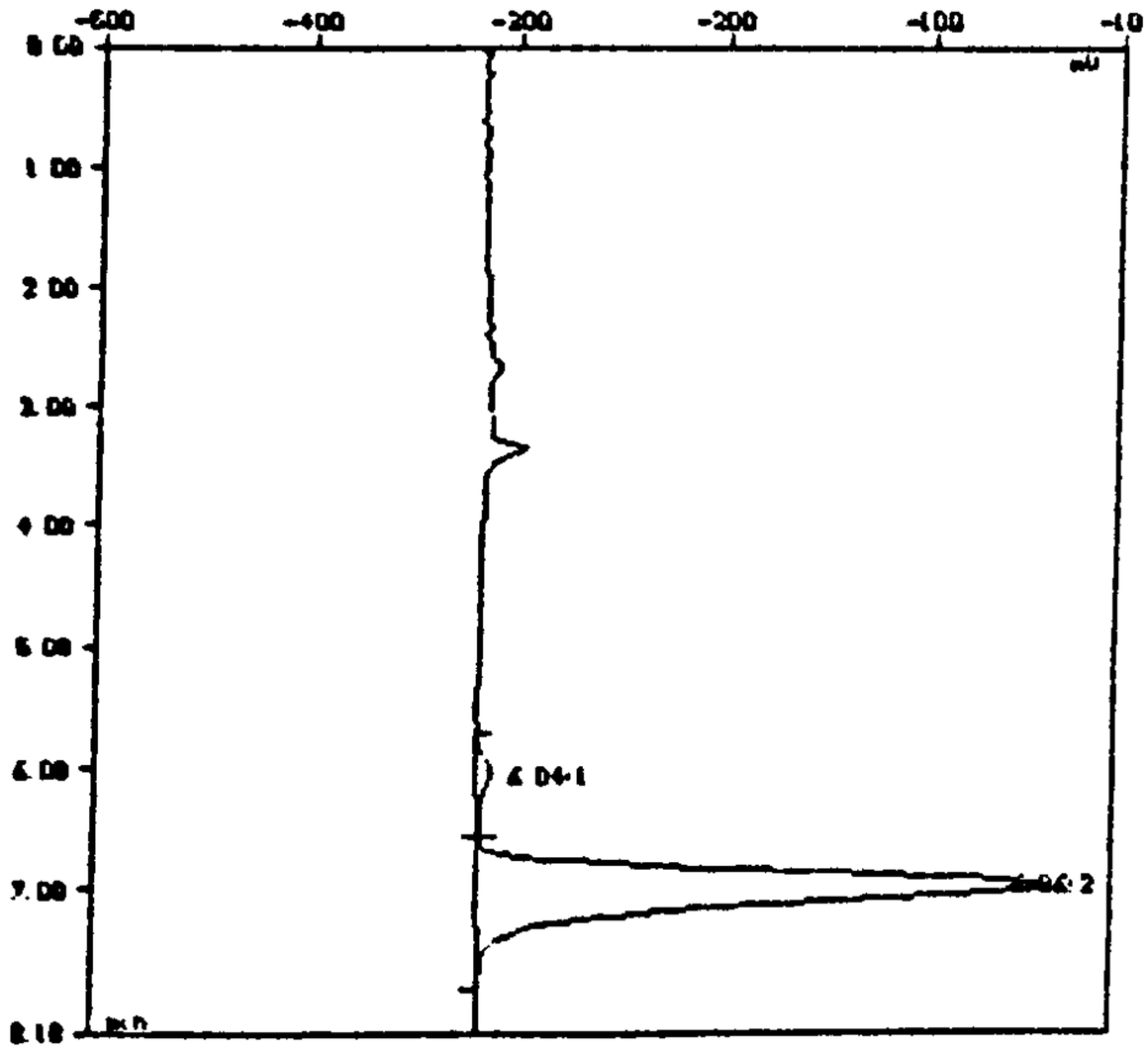


To a solution of allyl tetraglycolphthalimide **127** (1.0 g, 2.75 mmol) in ethanol (50 ml) hydrazine monohydrate was added dropwise (approximately 200 mg, 4.0 mmol, excess) over a period of 30 minutes at which point TLC (ethyl acetate:hexane, 2:3 and permanganate dip to visualise) indicated that the starting material had been consumed. Concentrated hydrochloric acid (10 drops) was carefully added, the reaction mixture was refluxed for 2 hours and then cooled to room temperature after which time TLC (methanol:dichloromethane, 1:9 and ninhydrin dip to visualise) indicated the presence of the product. The reaction mixture was then concentrated in *vacuo*, diluted with ethyl acetate (10 ml) and the product extracted with dilute hydrochloric acid (1 M, 2 x 5 ml). The aqueous extract was made basic with sodium hydroxide (4 M, 2 x 5 ml) and the product extracted with diethyl ether (4 x 10 ml). The organic fractions were combined and reduced under *vacuo* to give **130** as a clear pale yellow oil (436 mg, 1.87 mmol, 68%). ν_{max}(Nujol)/cm^{-1} 3401, 1652; δ_H(300 MHz; $CDCl_3$) 5.93 (1 H, ddt, *J* 17.3, 10.4, 5.7, CH_2CHCH_2O), 5.28 (1 H, ddt, *J* 17.3, 1.5, 1.5, *trans*-CH_2CHCH_2O), 5.18 (1 H, ddt, *J* 10.4, 1.5, 1.5, *cis*-CH_2CHCH_2O), 4.03 (2 H, dt, *J* 5.7, 1.5, CH_2CHCH_2O), 3.69-3.59 (16 H, m, CH_2), 2.85 (2 H, br s, NH_2); δ_C(75 MHz; $CDCl_3$) 135.1 (2 CH), 117.5 (CH_2), 73.8 (CH_2), 72.6 (CH_2), 71.0 (4 CH_2), 70.7 (CH_2), 69.8 (CH_2); *m/z* (CI) 234 (MH^+); (Found MH^+, 234.1707. $C_{11}H_{23}NO_4$ requires MH^+, 234.1705). COSY spectra exhibited a good correlation with the proposed structure.

Coupling **128** *to vancomycin*

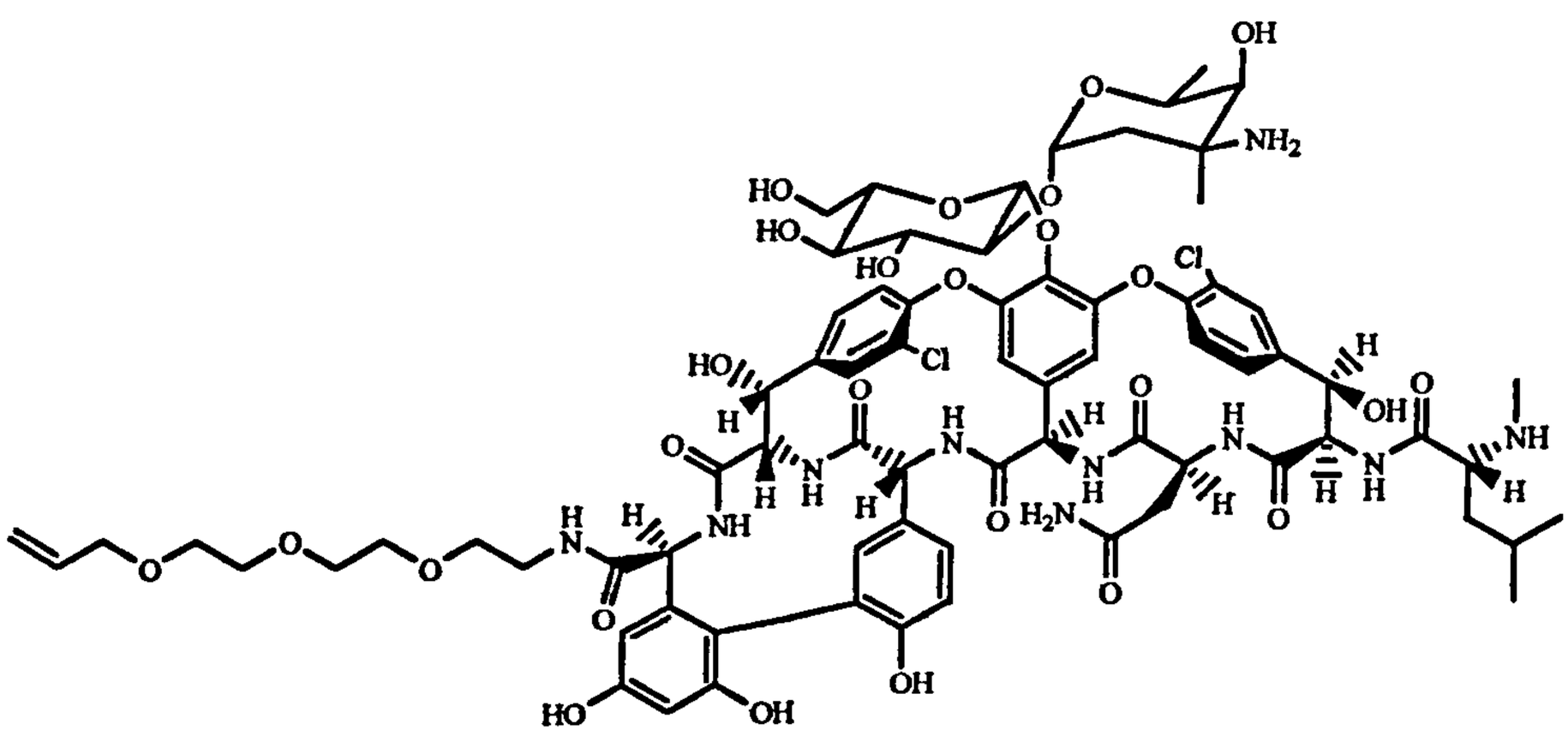


To a solution of vancomycin hydrochloride (50 mg, 33.6 μmol) in dimethyl sulfoxide was added allyl diethylglycolamine (20 mg, 138 μmol, excess), PyBOP (21 mg, 40.3 μmol, 1.2 eq), DIPEA (5.2 mg, 40.3 μmol,1.2 eq), HOBt (5.4 mg, 40.3 μmol, 1.2 eq). The reaction mixture was stirred at room temperature for 4 days after which time reverse phase HPLC indicated that the starting material had been consumed. The solution was concentrated under reduced pressure and the product isolated by preparative reverse phase HPLC to give **131** as a white powder (18 mg, 11.2 μmol, 33%). The mass spectrometry analysis is to follow. The reverse phase HPLC chromatogram is shown below. The starting materials vancomycin, PyBOP and HOBt were found to co-run giving a broad peak with retention times of 2.09 minutes (analytical column; mobile phase acetonitrile:water, 1:3; flow rate, 1 ml/minute; 24°C) and 3.48 minutes (analytical column; mobile phase TFA:acetonitrile:water, 1:24:75; flow rate, 1 ml/minute; 24°C).

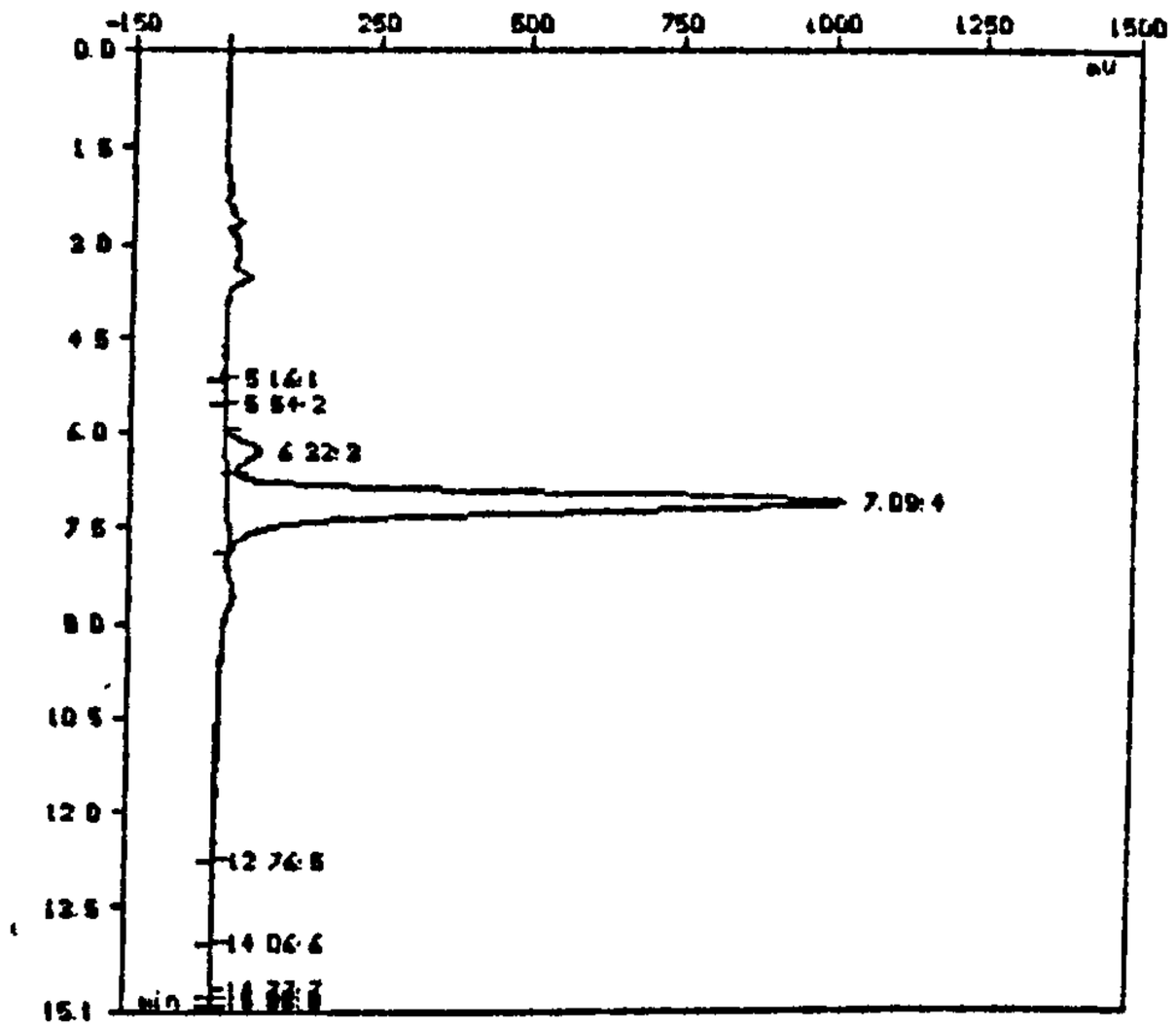


Analytical column; mobile phase TFA:acetonitrile:water, 1:24:75; flow rate, 1 ml/minute; 23°C.

Coupling **129** *to vancomycin*

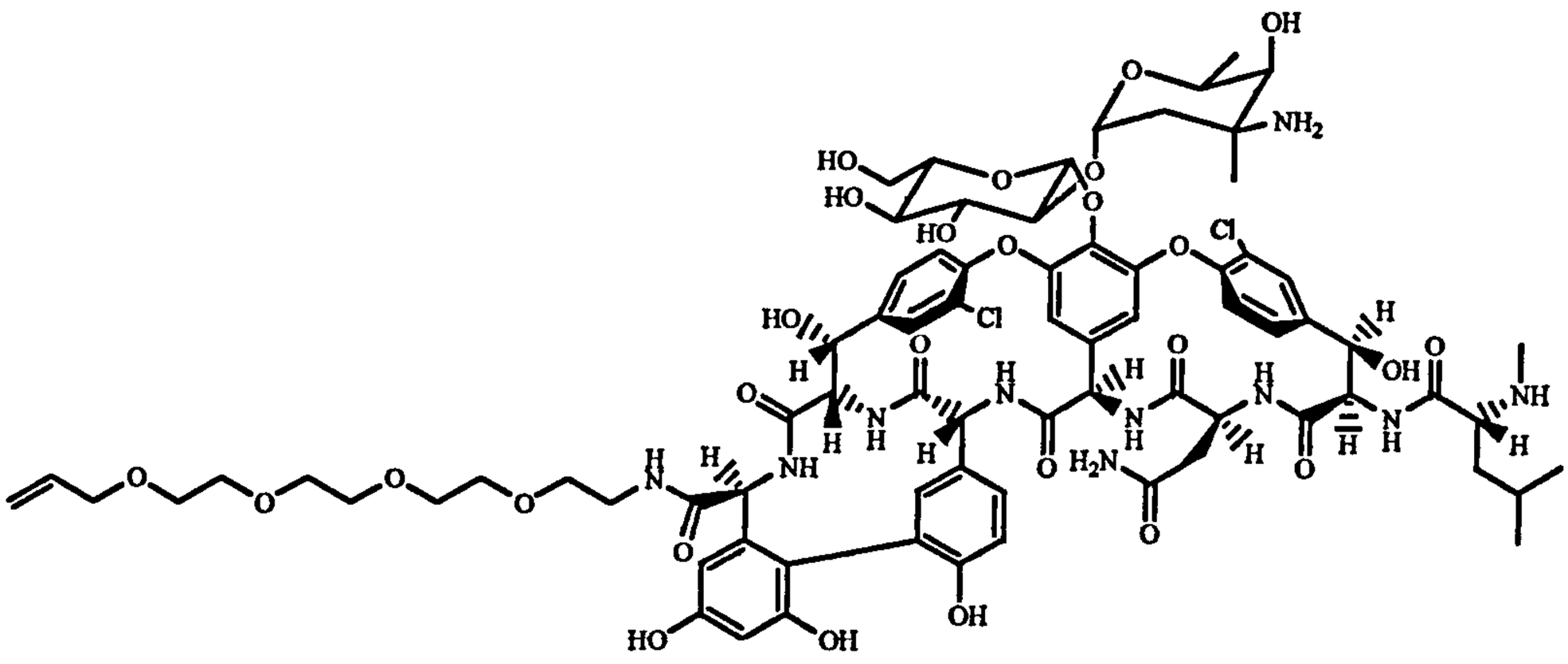


To a solution of vancomycin hydrochloride (50 mg, 33.6 μmol) in dimethyl sulfoxide was added allyl triethylglycolamine (20 mg, 106 μmol, excess), PyBOP (21 mg, 40.3 μmol, 1.2 eq), DIPEA (5.2 mg, 40.3 μmol,1.2 eq), HOBt (5.4 mg, 40.3 μmol, 1.2 eq). The reaction mixture was stirred at room temperature for 4 days after which time reverse phase HPLC indicated that the starting material had been consumed. The solution was concentrated under reduced pressure and the product isolated by preparative reverse phase HPLC to give **132** as a white powder (16 mg, 9.66 μmol, 29%); *m/z* (ES) 811 (MH_2^{2+}) 1621 (MH^+). The reverse phase HPLC chromatogram is shown below.

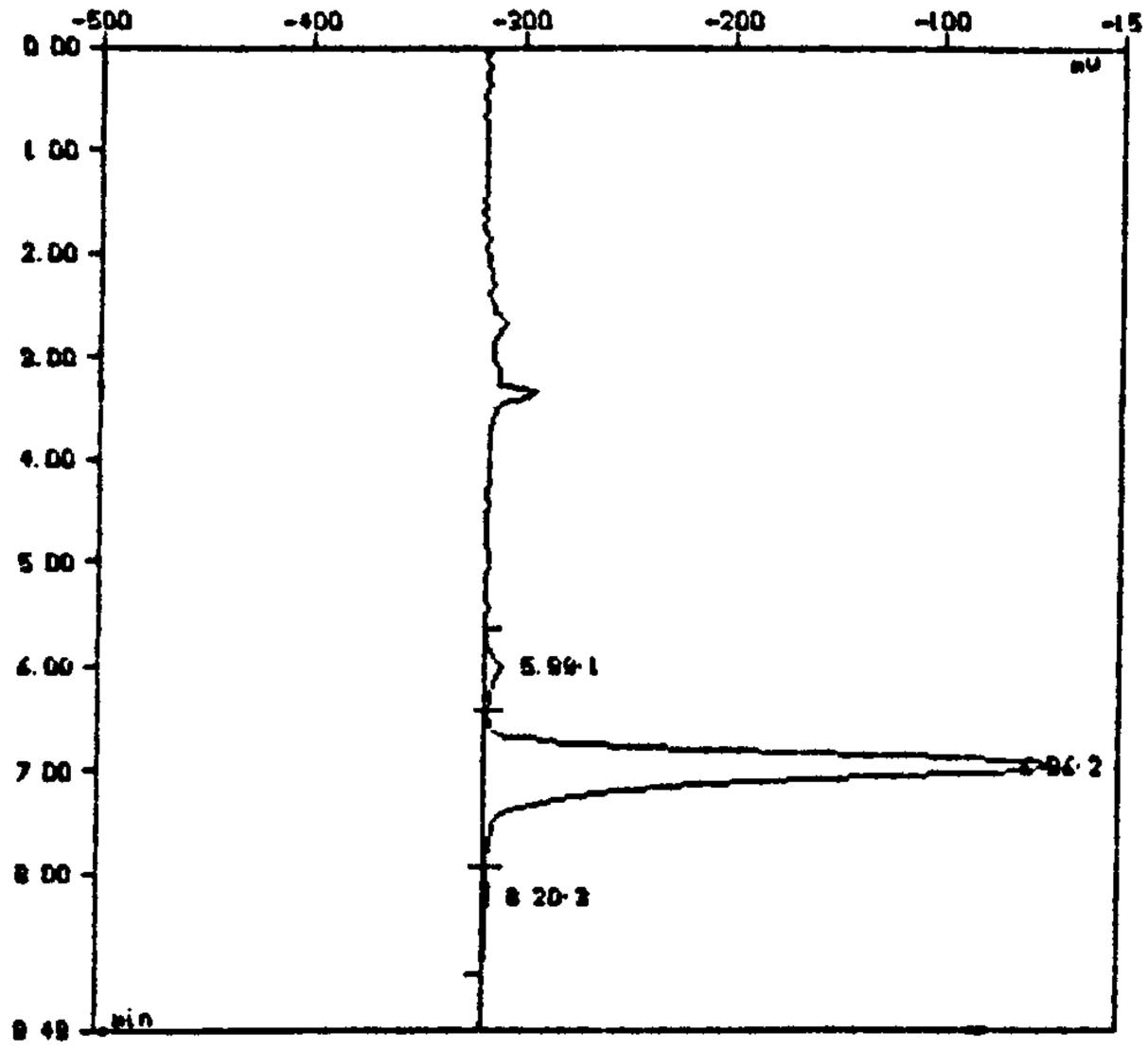


Analytical column; mobile phase TFA:acetonitrile:water, 1:24:75; flow rate, 1 ml/minute; 22°C; pressure 226 bar.

Coupling **130** *to vancomycin*

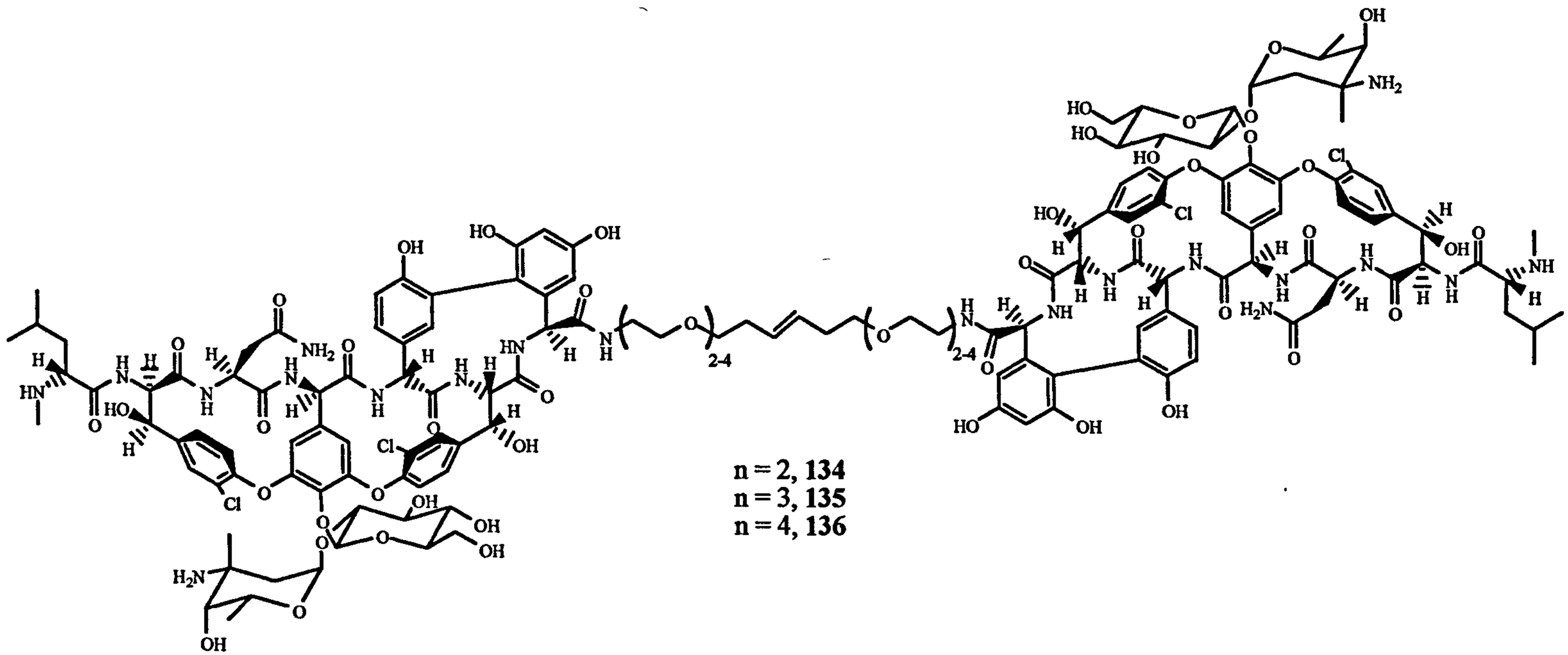


To a solution of vancomycin hydrochloride (50 mg, 33.6 μmol) in dimethyl sulfoxide was added allyl tetraethylglycolamine (20 mg, 86 μmol, excess), PyBOP (21 mg, 40.3 μmol, 1.2 eq), DIPEA (5.2 mg, 40.3 μmol,1.2 eq), HOBt (5.4 mg, 40.3 μmol, 1.2 eq). The reaction mixture was stirred at room temperature for 4 days after which time reverse phase HPLC indicated that the starting material had been consumed. The solution was concentrated under reduced pressure and the product isolated by preparative reverse phase HPLC to give **133** as a white powder (19 mg, 11.4 μmol, *34%*); *m/z* (ES) 833 (MH_2^{2+}). The reverse phase HPLC chromatogram is shown below.



Analytical column; mobile phase TFA:acetonitrile:water, 1:24:75; flow rate, 1 ml/minute; 24°C; pressure 205 bar.

Metathesis dimerisation to give **134-136.**[92, 93]



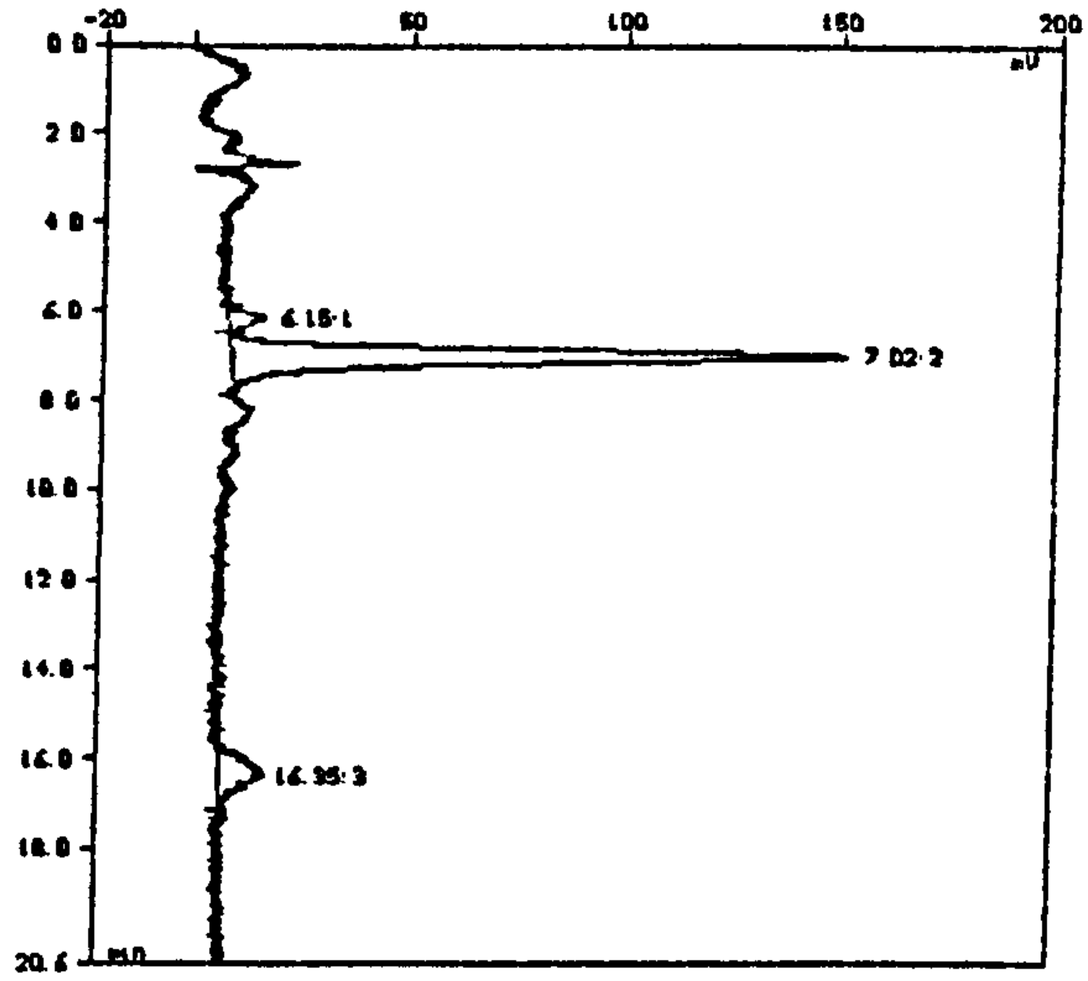
n = 2, 134
n = 3, 135
n = 4, 136

The general procedure for the vancomycin dimerisation *via* metathesis to give **134-136** was;

Dichloromethane (100 ml) was frozen in liquid nitrogen and degassed under reduced pressure 4 times. Grubbs catalyst (12.4 mg, 15 μmol) was added to this and dissolved over 20 minutes *via* ultra-sonification to give a pale red brown solution. The vancomycin monomer (1 mg, ~0.6 μmol) was added to the solution of Grubbs catalyst in dichloromethane (1 ml, 0.15 μmol, 25 mol%) and dissolved *via* ultra-sonification over a period of 20 minutes. The solution was frozen in liquid nitrogen and degass under reduced pressure 3 times then allowed to warm to room temperature. The reaction mixture was allowed to stir at room temperature for 2 days after which time analysis by reverse phase HPLC was undertaken. The reverse phase HPLC chromatograms are shown below.

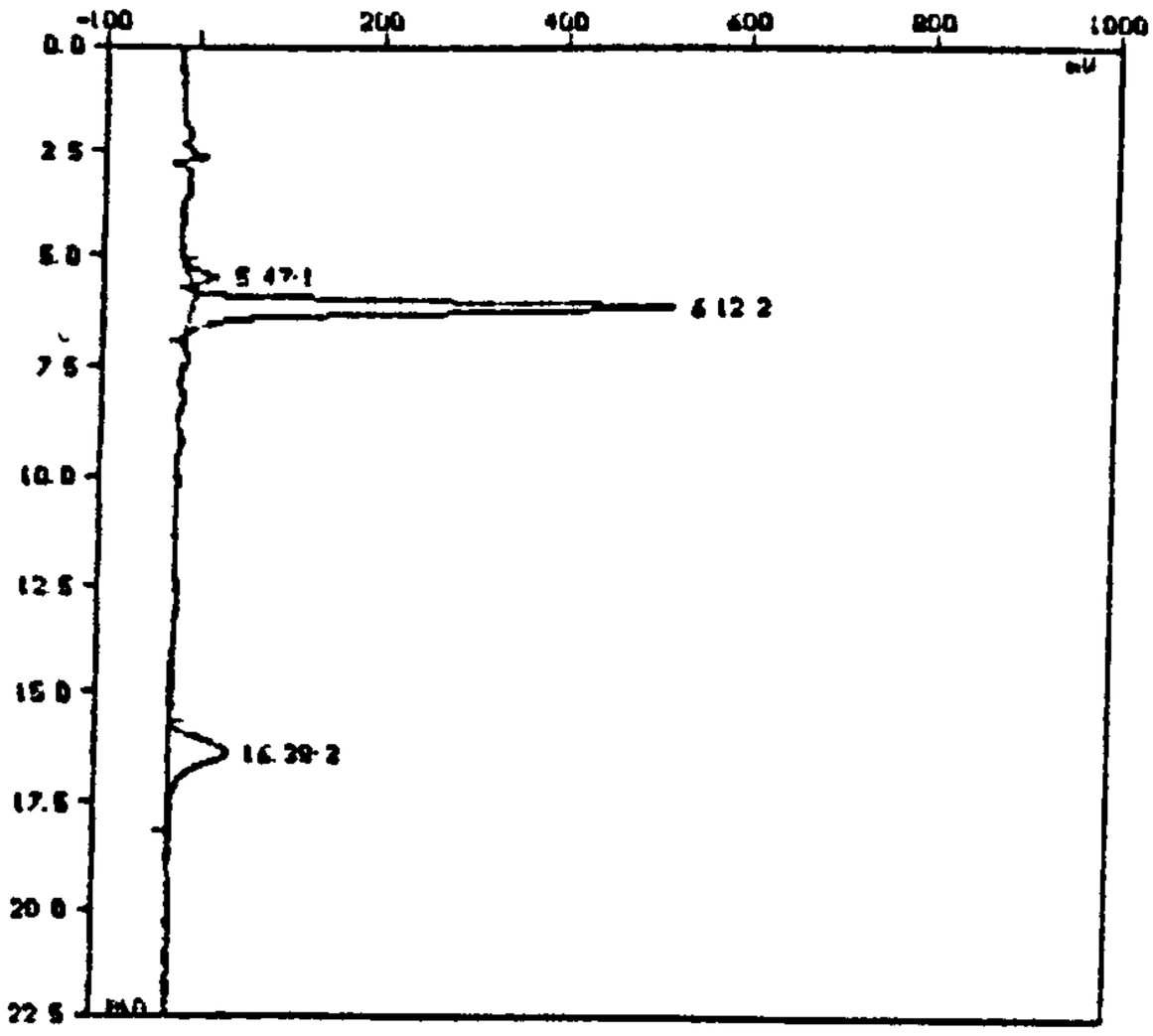
136; *m/z* (ES) 1081 (MH_3^{3+}), 1621 (MH_2^{2+}) (Found: M^+, 3237.1038. $C_{150}H_{184}{}^{35}Cl_4N_{20}O_{52}$ requires M^+, 3237.1123).

Analytical chromatogram of for **134**.



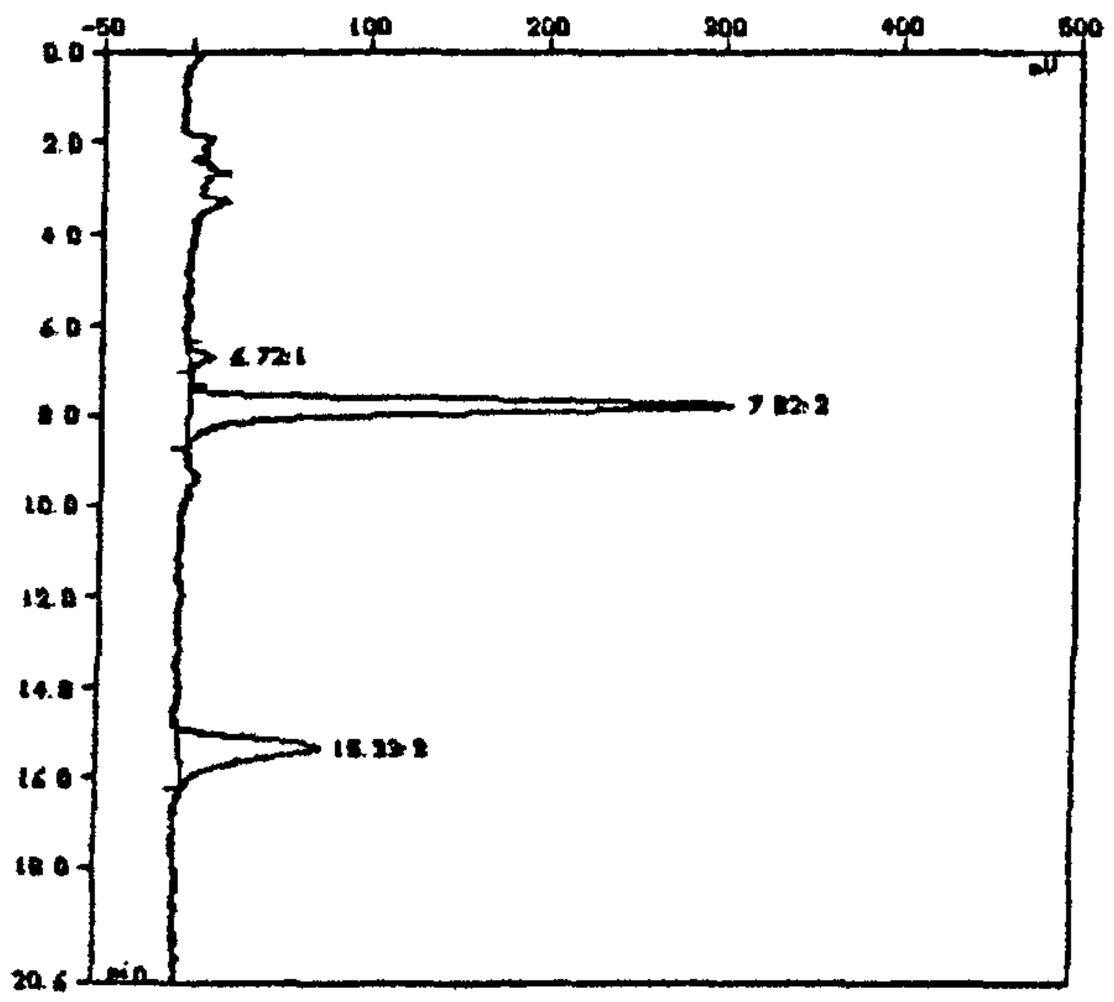
Analytical column; mobile phase TFA:acetonitrile:water, 1:24:75; flow rate, 1 ml/minute; 22°C; pressure 226 bar.

Analytical chromatogram for **135**.



Analytical column; mobile phase TFA:acetonitrile:water, 1:24:75; flow rate, 1 ml/minute; 22°C; pressure 226 bar.

Analytical chromatogram for **136**.



Analytical column; mobile phase TFA:acetonitrile:water, 1:24:75; flow rate, 1 ml/minute; 25°C; pressure 184 bar.

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